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**BOX PATENT APPLICATION**

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RE: *U.S. Patent Application Entitled: COMPOSITIONS AND METHODS OF  
MODULATING CHOLESTEROL METABOLISM – David J. Mangelsdorf, Joyce J.  
Repa, Stephen D. Turley and John M. Dietschy (UTSMC/DAL:596)*

Sir:

Transmitted herewith for filing is a 106-page patent specification including 58 claims and an abstract. Also included are Figures 1-10B on 10 sheets. The specification and drawings constitute the application of David J. Mangelsdorf, Joyce J. Repa, Stephen D. Turley and John M. Dietschy for the captioned invention.

Also transmitted herewith is a diskette containing the computer-readable form of those sequences in the specification, a Statement as Required Under 37 C.F.R. § 1.821(f), and a separate paper copy of the sequence listing.

Please note that this application is filed without an inventors' Declaration and Assignment, a Declaration Claiming Small Entity Status, a Power of Attorney, and filing fees. Pursuant to 37 C.F.R. § 1.53(b) and (f), the Applicants request the Patent and Trademark Office to accept this application and accord a serial number and filing date as of the date this application is deposited with the U.S. Postal Service for Express Mail. Further, the Applicants request that the NOTICE OF MISSING PARTS-FILING DATE GRANTED pursuant to 37 C.F.R. § 1.53(f) be sent to the undersigned Applicants' representative.

ARNOLD WHITE & DURKEE

Assistant Commissioner for Patents

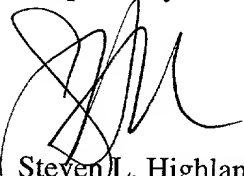
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Please forward any reply to this communication directly to our Houston office for docketing purposes. The mailing address and the physical address for courier packages is 750 Bering Drive, Houston, Texas, 77057-2198, and the Houston fax number is 713.787.1440.

Respectfully submitted,



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**PATENT**  
**UTSD:596**

**APPLICATION FOR UNITED STATES LETTERS PATENT**  
**for**  
**COMPOSITIONS AND METHODS OF MODULATING CHOLESTEROL**  
**METABOLISM**  
**by**  
**David J. Mangelsdorf**  
**Joyce J. Repa**  
**Stephen D. Turley**  
**and**  
**John M. Dietschy**

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## BACKGROUND OF THE INVENTION

The present application claims the benefit of U.S. Provisional Patent Application, Serial Number 60/111,894, filed December 10, 1998.

5

### A. Field of the Invention

The present invention relates generally to the fields of biochemistry and molecular biology. More particularly, it relates to lipid metabolism and the regulation of lipid metabolism. In a specific embodiment, it relates to inhibition of intestinal absorption of cholesterol by ligands that bind to the retinoid X nuclear hormone receptor (RXR).

10

### B. Description of Related Art

Sterols are structural lipids present in the membranes of most eukaryotic cells. These lipids are rigid and characterized by a four ring hydrocarbon steroid nucleus. Sterols are required not only to impart membrane fluidity, but also serve as the precursors for a variety of products with specific biological activities. For example, cholesterol, an amphipathic sterol with a polar hydroxyl head group and nonpolar hydrocarbon body (the steroid nucleus), is the major sterol found in animal tissues. Cholesterol is an essential molecule, playing a critical role in the structural integrity of cell membranes, a precursor for steroid hormones and serves as a precursor for bile acids. Cholesterol is synthesized in the liver from isoprene precursors and further obtained via dietary intake.

15

20

Although cholesterol is a requisite molecule, high levels of blood cholesterol or hypercholesterolemia has been implicated in atherosclerosis, heart attack, and stroke (Schultheis, 1990; Mitchell, 1990). Hypercholesterolemia, if not controlled, is one of several conditions that can lead to coronary artery disease. Coronary artery disease is the leading cause of death in the United States, accounting for approximately 600,000 deaths per year. Thus, the need exists for methods of treatment that can reduce cholesterol levels and methods to screen patients at risk for high cholesterol.

30



Possible targets for treatment are transcription factors involved in cholesterol metabolism. One such set of factors, nuclear receptors, are ligand-activated transcription factors that govern aspects of every major developmental and metabolic pathway (reviewed in Kastner *et al.*, 1995; Mangelsdorf *et al.*, 1995). For example, the LXRs were first identified as “orphan” members of the nuclear receptor superfamily whose ligands and functions are unknown (Willy and Mangelsdorf, 1998). The LXRs have recently been shown to be activated by a specific class of naturally occurring, oxidized derivatives of cholesterol, including 22(*R*)-hydroxycholesterol, 24(*S*)-hydroxycholesterol, and 24,25(*S*)-epoxycholesterol (Janowski *et al.*, 1996; Lehmann *et al.*, 1997). Oxysterols are concentrated in tissues where cholesterol metabolism and LXR expression are high, such as liver, brain, and placenta (Lavy *et al.*, 1977; Spencer *et al.*, 1985; Lütjohann *et al.*, 1996).

LXRs function as heterodimers with the retinoid X receptors (RXRs), and thus, the RXR/LXR complex can be activated by both RXR ligands (*i.e.*, rexinoids) and oxysterols (Teboul *et al.*, 1995; Willy *et al.*, 1995; Janowski *et al.*, 1996). Two LXR proteins ( $\alpha$  and  $\beta$ ) are known to exist in mammals. The expression of LXR $\alpha$  is restricted, with highest levels in the liver (hence, the name liver X receptor) and lower but significant levels in kidney, intestine, spleen, and adrenals (Apfel *et al.*, 1994; Willy *et al.*, 1995). LXR $\beta$  expression is more widespread and has been found in nearly every tissue examined (Shinar *et al.*, 1994; Song *et al.*, 1994).

The pattern of expression of LXRs and their oxysterol ligands first suggested that these receptors may have a role in cholesterol metabolism. Cholesterol has two essential metabolic fates in mammals: conversion into steroid hormones or bile acids. Since steroid hormone synthesis is known to be governed by the orphan nuclear receptor steroidogenic factor-1 (SF-1) (Parker and Schimmer, 1997), it is possible that LXRs are involved in bile acid synthesis (Janowski *et al.*, 1996). A likely target for any bile acid inducer is cholesterol 7 $\alpha$ -hydroxylase (Cyp7a), the rate-limiting enzyme in the classical bile acid synthesis pathway (Janowski *et al.*, 1996; Lehmann *et al.*, 1997). Experiments

by the inventors and others have shown that the *Cyp7a* promoter contains a functional LXR response element that can be activated by RXR/LXR heterodimers in an oxysterol- and retinoid-dependent manner (Lehmann *et al.*, 1997). The formation of bile acids is one of two major pathways for the catabolism and excretion of cholesterol in mammals (Russell and Setchell, 1992). Perturbations in this pathway may lead to a variety of disorders, including cholesterol gallstones, atherosclerosis, and some lipid storage diseases (Akiyoshi *et al.*, 1986; Turley and Dietschy, 1988; Carey and Duane, 1994). Together, these observations have raised an interesting possibility that LXRs may function as transcriptional control points in bile acid metabolism.

The RXR protein of RXR homo- and heterodimers has been observed to be regulated by 9-*cis* retinoic acid, which binds to the carboxy-terminus of RXR (Mangelsdorf and Evans, 1995). RXR can form heterodimers with numerous other proteins in the nuclear receptor superfamily, including LXR. Depending on the receptor protein that dimerizes with RXR, and the ligands present, the resulting effects of the heterodimer on transcription can vary. Synthetic retinoids have been found to selectively bind and activate RXRs (U. S. Patent 5,780,676 and U. S. Patent 5,455,265).

The potential to modulate lipid concentrations *in vivo*, by targeting proteins of the nuclear hormone receptor superfamily with specific ligands would be particularly useful in the treatment of various diseases related to lipid metabolism. For example, high blood cholesterol levels are associated with coronary disease. Lowering dietary cholesterol intake can significantly reduce cholesterol levels in most people. However, lowering dietary intake of cholesterol often is not enough, as certain individuals sustain high cholesterol blood levels due to inefficient endogenous cholesterol homeostasis. Thus, the ability to reduce blood cholesterol levels would prove to be extremely beneficial to these individuals. Currently, there are various drugs that are administered to treat hypercholesterolemia and other abnormal blood lipid levels. For example, cholestyramine and colestipol are resins that bind bile acids in the intestinal tract, causing the liver to increase its production of bile acids and thus lower the cholesterol levels, by

converting cholesterol into bile acid. However, the efficacy of these drugs is low, they are unpleasant to take, and result in constipation and bloating. Nicotinic acid, gemfibrizol, probucol, and lovastatin also are drugs used to lower blood lipid levels, but each has undesirable side effects associated with it. For example, probucol lowers total cholesterol levels, but also results in the undesirable lowering of HDL cholesterol.

Given the high incidence of coronary artery disease in the United States and its association with high cholesterol, there is a high demand for a treatment that can lower cholesterol levels without adverse side effects. Furthermore, a treatment that can lower LDL cholesterol levels, without affecting total lipid levels is highly desirable.

### **SUMMARY OF THE INVENTION**

It is, therefore, a goal of the present invention, to provide both compositions and methods that are related to cholesterol metabolism and abnormalities therein. More particularly, the invention is intended to provide tool for the identification of compositions for use in treating cholesterol-related pathologies, as well as therapeutic methods of use.

Thus, in a first embodiment, the present invention provides a non-human transgenic mammal, the cells of which comprise at least one non-functional endogenous LXR $\alpha$  allele. The transgenic mammal may further comprise two non-functional endogenous LXR $\alpha$  alleles. The mammal of the present invention may a mouse, rat, hamster, guinea pig, rabbit, cow, and sheep, or other suitable organism.

In particular embodiments, either one or both non-functional LXR $\alpha$  allele may contain (a) an interruption in the LXR $\alpha$  coding sequence, (b) a nonsense mutation in the LXR $\alpha$  coding sequence or (c) a deletion of LXR $\alpha$  coding sequence. Alternatively, the non-functional LXR $\alpha$  allele may result from an alteration in the regulatory region of the LXR $\alpha$  gene. In a particular embodiment, substitution of the endogenous an

inducible/repressable promoter for the endogenous LXR $\alpha$  promoter may be utilized. Cells of the transgenic mammal may further comprise an exogenous selectable marker gene under the control of a promoter active in at least one mammalian cell type.

5 Another aspect of the present invention provides a method for screening an RXR agonist or LXR $\alpha$  agonist candidate substance for the ability to increase bile acid synthesis comprising: 1) providing a cell; 2) contacting the cell with the candidate substance; and  
10 3) monitoring a bile acid-related phenotype of the cell, wherein an increase in the bile acid-related phenotype in the cell treated with the candidate substance, as compared to a similar cell not treated with the candidate substance, indicates that the candidate substance increases bile acid synthesis. In one embodiment of the present invention the cell is a liver cell. The bile acid-related phenotype may be expression of a gene involved in bile acid synthesis, for example, *Cyp7a*. The candidate substance may be an RXR agonist, more particularly a rexinoid.

15 Also contemplated in the present invention is a method for screening a candidate substance for the ability to reduce cholesterol levels in a mammal comprising: 1) providing a non-human transgenic mammal, the cells of which comprise at least one non-functional endogenous LXR $\alpha$  allele; 2) treating the mammal with the candidate  
20 substance; and 3) monitoring a cholesterol-related phenotype in the mammal, wherein a reduction in the cholesterol-related phenotype in mammals treated with the candidate substance, as compared to a similar mammal not treated with the candidate substance, indicates that the candidate substance reduces cholesterol levels. The mammal may be selected from the group consisting of mouse, rat, hamster, guinea pig, rabbit, cow, and  
25 sheep. The cells of the mammal may comprise two non-functional endogenous LXR $\alpha$  alleles.

The phenotype may be cholesterol absorption, circulating cholesterol, hepatic cholesterol, hepatomegaly, atherosclerosis, cardiac failure, cardiac (atrophy/hypertrophy),  
30 activity level, survival, cancer, reproduction, immune function, skin disease, cognitive

function, and adrenal function. The mammal may be maintained on a high cholesterol diet. The mammal may further be treated with an agent that blocks cholesterol biosynthesis.

5 Also contemplated is a method for screening a candidate substance for the ability to increase bile acid synthesis in a mammal comprising: 1) providing a non-human transgenic mammal, the cells of which comprise at least one non-functional endogenous LXR $\alpha$  allele; 2) treating the mammal with the candidate substance; and 3) monitoring a  
10 bile acid-related phenotype in said mammal wherein an increase in the bile acid-related phenotype in mammals treated with the candidate substance, as compared to a similar mammal not treated with the candidate substance, indicates that the candidate substance increases bile acid synthesis. The mammal may be selected from the group consisting of mouse, rat, hamster, guinea pig, rabbit, cow, and sheep. The bile acid-related phenotype may be selected from the group consisting of cholesterol level, Cyp7a synthesis, fecal bile  
15 acid excretion, bile acid pool size and bile acid composition.

Another aspect of the present invention is a method for screening a rexinoid for the ability to inhibit cholesterol absorption by an intestinal cell comprising: 1) providing an intestinal cell; 2) treating the cell with the rexinoid; and 3) monitoring cholesterol  
20 absorption by the cell, wherein a reduction in cholesterol absorption by the cell treated with the rexinoid, as compared to a similar cell not treated with the rexinoid, indicates that the rexinoid is an inhibitor of cholesterol absorption. The cell may be a duodenal cell, optionally located in a mammal. The method may further comprise comparing the effect of the candidate substance on cholesterol absorption on a cell comprising one or  
25 two non-functional endogenous LXR $\alpha$  alleles.

Another aspect of the present invention is a method of reducing cholesterol levels in a mammal comprising the step of treating the mammal with an RXR agonist. The agonist may be a rexinoid. The method may further comprise treating the mammal with  
30 an agent that inhibits cholesterol biosynthesis, for example, an HMG CoA reductase

inhibitor. The mammal may be a human. The method may further comprise stimulating bile acid synthesis in the mammal or reducing cholesterol intake by the mammal.

In yet another aspect of the present invention, there is provide a method for  
5 inhibiting cholesterol absorption in a mammal comprising treating the mammal with an RXR agonist. The agonist may be a rexinoid and the mammal may be human

The present invention further contemplates a transgenic cell which comprises at  
least one non-functional endogenous LXR $\alpha$  allele. Additionally, the transgenic cell may  
10 comprises two non-functional endogenous LXR $\alpha$  alleles.

Also contemplated is a rexinoid compound that inhibits cholesterol absorption,  
identified by a process comprising: 1) providing an intestinal cell; 2) treating the cell with  
a rexinoid; and 3) monitoring cholesterol absorption by the cell, wherein a reduction in  
15 cholesterol absorption by the cell treated with the rexinoid, as compared to a similar cell  
not treated with the rexinoid, identified the rexinoid as an inhibitor of cholesterol  
absorption.

Additionally, the present invention provides a rexinoid compound that inhibits  
20 cholesterol absorption, produced by a process comprising: 1) providing an intestinal cell;  
2) treating the cell with a rexinoid; 3) monitoring cholesterol absorption by the cell,  
wherein a reduction in cholesterol absorption by the cell treated with said rexinoid, as  
compared to a similar cell not treated with a rexinoid, identified the rexinoid as an  
inhibitor of cholesterol absorption; and 4) producing said rexinoid compound.

25

The present invention also provides a method of screening for a modulator of  
ABC1 expression comprising: 1) providing a cell expressing an RXR; 2) contacting said  
cell with a rexinoid and a candidate substance; and 3) determining the expression of  
ABC1 in said cell, wherein a change in expression of ABC1, as compared to a cell of step  
30 (b), indicates that said candidate substance is a modulator of ABC1 expression. Screening

for a modulator of ABC1 expression further comprises the step of determining the expression of ABC1 in a cell expressing RXR in the absence of said candidate substance. ABC1 expression is measured by RNA analysis, such as Northern analysis or PCR. Alternatively, ABC1 expression is measured by protein analysis, such as ELISA or Western blot. In preferred embodiments, screening for a modulator of ABC1 expression in a cell comprises an exogenous marker cassette comprising a polynucleotide encoding a screenable marker operably linked to an ABC1 promoter region. In preferred embodiments, the screenable marker is an esterase, phosphatase, protease, green fluorescent protein, luciferase, chloramphenicol acetyl transferase,  $\beta$ -galactosidase,  $\beta$ -glucuronidase or a drug resistance marker. Preferred cells for screening for a modulator of ABC1 expression are cells expressing an RXR such as intestinal cells, preferably of duodenal or jejunal origin. Screening for a modulator of ABC1 expression may be done *in vivo*.

The present invention also provides for a method of making a modulator of ABC1 expression comprising: 1) providing a cell expressing an RXR; 2) contacting said cell with a rexinoid and a candidate substance; 3) determining the expression of ABC1 in said cell, wherein a change in expression of ABC1, as compared to a cell of step (b), indicates that said candidate substance is a modulator of ABC1 expression; and 4) making said modulator.

### **BRIEF DESCRIPTION OF THE DRAWINGS**

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

**FIG. 1 - Liver Accumulation of Cholesterol in LXR $\alpha$  Wild-Type and Knock-Out Mice Treated with LG268.** Values represent mean  $\pm$  SEM for 6 animals per group. LG268 given at 30 mg/kg body weight for 10 days.

5 **FIG. 2A and FIG. 2B - Comparison of Liver Cholesterol Levels with Bile Acid Production in LXR $\alpha$  Wild-Type and Knock-Out Mice Treated with LG268.** Open bars represent mice fed control diet; closed bars represent mice fed diet containing 2% cholesterol. Values represent mean  $\pm$  SEM for 6 mice per group. LG268 given at 30 mg/kg body weight for 7 days. Veh = Vehicle alone.

10

**FIG. 3 - Cholesterol Absorption in LXR $\alpha$  Wild-Type and Knock-Out Mice Treated with LG268.** Values represent mean  $\pm$  for 6 mice per group. LG268 given at 30 mg/kg body weight for 10 days.

15 **FIG. 4 - Dose Response Curve for LG268 Inhibition of Cholesterol Absorption in LXR $\alpha$  Wild-Type Mice.** Values represent mean  $\pm$  SEM for 5 mice per group. LG268 was given at the indicated dose for 10 days.

20 **FIG. 5 - Lipid Absorption in LXR $\alpha$  Wild-Type Mice Treated with LG268.** Values represent mean  $\pm$  for 5 mice per group. LG268 given at 30 mg/kg body weight for 10 days.

25 **FIG. 6 - Comparison of Different Rexinoids for the Ability to Reduce Liver Cholesterol and Cholesterol Absorption.** Values represent the mean  $\pm$  SEM for 5 mice per group. LG268 and LG721 given at 3 mg/kg body weight for 10 days.

30 **FIG. 7 - Cholesterol Production in LXR $\alpha$  Wild-Type Mice Treated with LG268.** Values represent mean  $\pm$  SEM for 5 mice per group. LG268 given at 3 or 30 mg/kg body weight for 7 days.



**FIG. 8 - Prevention of Liver Cholesterol Accumulation and Cholesterol Absorption by LG268 in Golden Syrian Hamsters.** Values represent mean  $\pm$  SEM for 6 hamsters per group, except for surfomer treatment, which contained 5 hamsters.

**FIG. 9 - Expression of Cholesterol-Related Genes in Duodenal Intestine Samples Treated with LG268.** Values represent mean  $\pm$  SEM for 5 mice per group. LG268 was given at the indicated dose for 10 days. Northern analysis was performed on duodenal intestinal samples.

**FIG. 10A and FIG. 10B. - Expression of ATP-Binding Cassette Protein-1 in Duodenal Intestine Samples Treated with LG268.** Northern analysis was performed on intestinal samples following a 6 or 12 hour treatment with LG268 (30 mg/kg body weight). Northern gels were quantified using a phosphorimager, standardized against controls, and mathematically to yield 1 unit for the vehicle-treated control group.

## DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

Although cholesterol is an essential molecule required for the structural integrity of cell membranes, as a precursor for steroid hormones and bile acids, high levels of blood cholesterol can be detrimental. Hypercholesterolemia has been implicated in coronary artery disease (*i.e.*, atherosclerosis, heart attack, and stroke) (Schultheis, 1990; Mitchell, 1990). Coronary artery disease is the leading cause of death in the United States, accounting for approximately 600,000 deaths per year. Thus, the need exists for methods of treatment that can reduce cholesterol levels and methods to screen patients at risk for high cholesterol.

The present invention seeks to ameliorate high blood cholesterol levels by stimulating the RXR/LXR $\alpha$  hormone receptor. Rexinoids have been shown to bind to the RXR receptor, but not in any cholesterol-regulatory fashion. However, LXR $\alpha$  does have a putative relationship to cholesterol metabolism through bile acid metabolism. Since

LXR $\alpha$  functions as a heterodimer with the retinoid X receptor (RXR, Willy *et al.*, 1995) the inventors sought to determine if RXR agonists (rexinoids) also could affect cholesterol metabolism.

5 Initial experiments utilized the RXR-specific ligand, LG100268 (hereafter referred to as LG268), which binds and activates all RXR subtypes and fails to exhibit binding to other related nuclear receptors (Boehm, 1995). Studies were performed in wild-type and LXR $\alpha$ -knockout mouse strains and in Syrian golden hamsters. LG268 produced dramatic effects on cholesterol homeostasis in mice and hamsters by preventing  
10 the liver accumulation of cholesterol typically seen in cholesterol-fed animals. This effect did not involve LXR $\alpha$ , since it was observed in the LXR $\alpha$  knockout mouse strain, nor did it occur by modulation of bile acid biosynthesis to eliminate ingested cholesterol, since the homozygote knockout could not bile acid synthesis. Rather, LG268 inhibited the intestinal absorption of cholesterol in a specific, dose-dependent manner with  
15 remarkable potency (effective at a dose of 1.4 mg/kg body weight which is equivalent to .0015% of the diet in mouse experiments). Absorption of other lipids appeared to be unaffected. As further evidence for the efficacy of rexinoids in blocking cholesterol absorption, even when mice were fed a diet containing 0.2% cholesterol (ten times what their basal diet provides), LG268 effected a total block in cholesterol absorption and the  
20 treated mice exhibited dramatically increased tissue cholesterol synthesis rates in order to meet their cholesterol needs.

Thus, LG268 and rexinoids provide powerful tools to elucidate the mechanism of cholesterol absorption and homeostasis. Practical applications include the use of these  
25 agents alone, or in conjunction with other anti-hypocholesterolemic agents such as the statins, niacin, or bile acid sequestrants (surfomer) to prevent the elevated blood cholesterol levels associated with atherosclerosis and heart disease.

## A. CHOLESTEROL METABOLISM

Most cholesterol synthesis in animals takes place in the liver.. The 5-carbon isoprene unit is the key intermediate in cholesterol biosynthesis. Cholesterol, is made from acetyl-CoA in four steps (Rao, 1995; Bocan *et al.*, 1998). The first step is the condensation of three acetate units catalyzed by thiolase, HMG-CoA synthase, and HMG-CoA reductase, resulting in the formation of the molecule mevalonate. Second, mevalonate is converted into activated isoprene units through a series of phosphorylation steps. Third, the 5-carbon isoprene units are polymerized into a 30-carbon linear structure of squalene through a series of enzyme catalyzed reactions. Finally, the squalene chain is cyclized into the four rings of the steroid nucleus and through further enzymatic steps of oxidation and demethylation, the final product of cholesterol is formed. Cholesterol can also be catabolized into oxysterols or converted into bile acids or steroid hormones, which is regulated by a cholesterol homeostasis feedback mechanism (Osborne and LaMorte, 1998)

Only a small percentage of the cholesterol synthesized in the liver is incorporated into the membranes of hepatocytes. The majority of the cholesterol made in the liver is exported to other tissues as either bile acids or cholesterol esters. While bile acids and bile salts are relatively hydrophilic cholesterol derivatives, aiding in lipid digestion, cholesterol esters are more hydrophobic and are formed in the liver by the enzyme acyl-CoA-cholesterol acyl transferase (ACAT). ACAT catalyzes the transfer of fatty acids from coenzyme A to the hydroxy group of cholesterol, which converts the cholesterol into a more hydrophobic molecule. Cholesterol esters are stored in the liver or transported to other tissues. The movement of cholesterol and cholesterol esters from the liver to tissues where they will be stored or utilized, require special carrier proteins (apolipoproteins) due to cholesterol's relative water insolubility. These apolipoproteins, form aggregates with cholesterol and other lipids such as phospholipids and triacylglycerides, to form the carrier complexes known as plasma lipoproteins.

## B. RXR HETERODIMERS

It has been assumed that receptors such as the TR and RAR, like the steroid receptors, function as homodimers. While the TR homodimer can be formed (Forman *et al.*, 1989; Wahlström *et al.*, 1992), an accessory factor present in nuclear extracts was required for high affinity binding of VDR, TR, and RAR to their cognate HREs (reviewed by Glass, 1994). Shortly after the identification of RXR (Mangelsdorf *et al.*, 1990), a series of studies in several laboratories produced the consensual finding that RXR was the common missing factor (Yu *et al.*, 1991; Kliewer *et al.*, 1992a; 1992b; Leid *et al.*, 1992b; Zhang *et al.*, 1992; Marks *et al.*, 1992; Bugge *et al.*, 1992). There now are known to be three mammalian RXR isoforms, and, remarkably, these are the only proteins that can subserve this function (Mangelsdorf *et al.*, 1992; Leid *et al.*, 1992b). In *Drosophila*, the RXR homolog Ultraspiracle is a partner for the ecdysone receptor, indicating that heterodimerization evolved prior to the divergence of vertebrates and invertebrates.

Because DRs are asymmetric, heterodimer complexes should bind these elements in an asymmetric fashion. Indeed, it now has been established that on DR3, DR4, and DR5, RXR occupies the 5' half-site and the partner (*e.g.*, VDR, TR, and RAR, respectively) occupies the 3' half-site (Perlmann *et al.*, 1993; Kurokawa *et al.*, 1993; Zechel *et al.*, 1994). On DR1, RXR can bind as both a homodimer and as a heterodimer with RAR. Interestingly, the polarity of the RXR/RAR heterodimer on DR1 is reversed (Kurokawa *et al.*, 1994). In many cell types, the consequence of this reverse polarity binding is that the RAR/RXR heterodimer is a potent repressor of the ligand-activated RXR homodimer (Mangelsdorf *et al.*, 1991; Kurokawa *et al.*, 1995).

Unexpectedly, the ligand-induced transcription activities for RXR are suppressed when complexed with VDR, TR, and RAR. In RXR/TR and RXR/RAR, the formation of the heterodimer actually prevents the RXR from binding its ligand (Kurokawa *et al.*, 1994; Forman *et al.*, 1995c). The restriction of RXR ligand binding within several of these heterodimers indicates that 9-*cis* retinoic acid responsiveness is not an obligatory consequence of heterodimerization with RXR. Thus, in these instances, RXR is said to

be a silent partner. Nonetheless, there are several examples in which RXR can be an active partner (reviewed by Leblanc and Stunnenberg, 1995). One example occurs in the PPAR/RXR heterodimer, in which both receptors are independently responsive and are synergistically activated in the presence of both ligands (Kliewer *et al.*, 1992b).  
5 Similarly, heterodimeric complexes of RXR with the orphan receptor LXR or farnesoid X receptor (FXR) also retain 9-*cis* retinoic acid responsiveness, further supporting the view that RXR can either be a silent partner or a hormone responsive partner (Willy *et al.*, 1995; Forman *et al.*, 1995a). The final and perhaps most unusual example of RXR responsiveness occurs in the complex formed with the orphan receptor NGFI-B  
10 (Perlmann and Jansson, 1995; Forman *et al.*, 1995c). This receptor has been shown to contribute to T cell apoptosis and is capable of binding to target DNA as a monomer. However, in the presence of RXR, it forms a stable heterodimer that is 9-*cis* retinoic acid responsive. Thus, RXR is a critical component of heterodimer formation, which in turn is critical to generating diversity of hormone responses.

### 15 C. ABC TRANSPORTER AND ABC-1 GENE

The ATP-binding cassette (ABC) transporters, also called the "traffic ATPases", comprise a superfamily of membrane proteins that mediate transport and channel functions in prokaryotes and eukaryotes (Higgins, 1992). ABC proteins share a similar  
20 overall structure and significant sequence homology. All ABC proteins contain a conserved domain of approximately two hundred amino acid residues which includes one or more nucleotide binding domains. A majority of these proteins are involved in active transport of molecules across membranes. Eukaryotic ABC proteins include: P-glycoproteins, also known as multidrug resistance (MDR) proteins, which are associated  
25 with resistance to a wide range of hydrophobic drugs (MDR1; Gottesman, Pastan, 1993) or with phosphatidylcholine transport (MDR2; Ruetz, Gros, 1994); CFTR, the cystic fibrosis transmembrane conductance regulator (Welsh, Smith, 1993); TAP proteins, the transporters associated with antigen processing in mammalian cells (Androlewicz *et al.*, 1994); cMOAT/cMRP1, which is associated with transport of glutathione, glucuronide,  
30 and sulfate conjugates across the canalicular membrane (Buchler *et al.*, 1996); and STE6,

which exports the a-factor mating pheromone of *S. cerevisiae* (Michaelis, 1993). Prokaryotic ABC proteins include periplasmic nutrient permeases, such as those responsible for uptake of maltose (MalFGK) and histidine (HisMPQ) in gram-negative bacteria, and toxin exporters such as those required for export of hemolysin (HlyB) and colicin (ColV) from *E. coli* (Higgins, 1992).

Savary *et al.* (1997) recently identified a novel ABC transporter, denoted ABC7, in mouse. The predicted 629 amino acid mouse ABC7 translation product contains six putative transmembrane domains near the N-terminus, followed by an ATP-binding cassette domain. Savary *et al.* (1997) also disclosed a partial protein sequence from human similar to the C-terminal 340 amino acids of mouse ABC7 protein. Savary *et al.* reported that the human ABC7 was widely expressed in cell lines, heart, skeletal muscle, pancreas, lung, liver, and placenta. Human ABC7 expression was not detected in brain.

Mutations in other ABC family members cause a variety of genetic disorders, such as cystic fibrosis, intrahepatic cholestasis, macular degeneration, peroxisomal dysfunction, and immunodeficiency syndromes (Higgins, 1992). The first clue that ABC1 participated in lipid metabolism came with the observation by Schmitz and colleagues that ABC1 expression rose when the cholesterol content of macrophages was increased (Langmann *et al.*, 1999). In an independent study using expression microarrays, Lawn *et al.* identified the ABC1 mRNA as an upregulated transcript in cholesterol-loaded macrophages (Lawn *et al.*, 1999). Macrophages routinely ingest large amounts of cholesterol in the form of cellular membrane debris and aggregated lipoproteins, and lack the ability to downregulate cholesterol uptake in response to cholesterol loading (Brown and Goldstein, 1983). Consequently, macrophages are heavily dependent on cholesterol efflux (predominantly through transfer to HDL) to prevent accumulation of lipids. Thus, it might be anticipated that proteins that participate in cholesterol efflux, such as ABC1, would be increased in cholesterol-laden macrophages. The current finding of Lawn *et al.* that ABC1 is located on the cell surface suggests that this transporter may actually physically participate in the hand-off of cholesterol from cells to plasma HDL (Lawn *et*

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al., 1999). A direct role for ABC-1 in cholesterol homeostasis came recently when 3 groups identified the underlying molecular defect of Tangier disease to be mutations in the ABC1 gene (Bodzioch *et al.*, 1999; Rust *et al.*, 1999; Brooks Wilson *et al.*, 1999). Lawn *et al.* extends these findings and provides a further functional characterization of the ABC1 transporter (Lawn *et al.*, 1999). These findings dovetails nicely with the previous observation that Tangier fibroblasts have a greatly reduced capacity to donate cellular cholesterol (and phospholipids) to apo A-I, the major apolipoprotein of HDL (Francis *et al.*, 1995). The full complement of substrates transported by ABC1 has not been identified, and it is possible that some of the variability in the clinical manifestations of this disease is due to mutation-specific differences in substrate specificity.

#### D. TRANSGENIC ANIMALS AND CELLS

Animal models serve as useful vehicles for screening of compounds, for example, for effects on cholesterol levels, cholesterol absorption, and bile acid synthesis. Transgenic animals, defined as animals with engineered genomes, may prove particularly useful in certain applications. In the present application, the focus is on regulation of cholesterol metabolism and the role of LXR $\alpha$  in this process. Thus, transgenic mice were produced that lacked either one, or both, of the endogenous LXR $\alpha$  alleles. Animals lacking one or both alleles showed increases in cholesterol levels, enlarged livers and reduced bile acid secretion. These animals have proved useful not only in elucidating the biology of this signalling pathway, but as tools in examining possible therapeutic compositions and methods of treatment.

Transgenic non-human animals of the present invention will have at least one non-functional endogenous LXR $\alpha$  allele. It further is contemplated that the transgenic non-human animal of the present invention may have both LXR $\alpha$  alleles altered, thus providing cells which lack functional LXR $\alpha$ . The non-functional LXR $\alpha$  allele(s) can contain an interruption of the LXR $\alpha$  coding sequence, a nonsense mutation that truncates the LXR $\alpha$  product, a deletion of the LXR $\alpha$  coding sequence or alterations in the LXR $\alpha$  regulatory region. In designing a heterologous gene for expression in animals, sequences

which interfere with the efficacy of gene expression, such as coding sequences, promoters, introns, polyadenylation signals, polymerase II termination sequences, hairpins, consensus splice sites and the like, may be eliminated.

5 Current advances in transgenic approaches and techniques have permitted the manipulation of a variety of animal genomes via gene addition, gene deletion, or gene modifications (Franz *et al.*, 1997). For example, mosquitos (Fallon, 1996), trout (Ono *et al.*, 1997), zebrafish (Caldovic and Hackett, 1995), pigs (Van Cott *et al.*, 1997) and cows (Haskell and Bowen, 1995), are just a few of the many animals being studied by  
10 transgenics. The creation of transgenic animals that express human proteins such as  $\alpha$ -1-antitrypsin, in sheep (Carver *et al.*, 1993); decay accelerating factor, in pigs (Cozzi *et al.*, 1997); and plasminogen activator, in goats (Ebert *et al.*, 1991) have previously been demonstrated. The transgenic synthesis of human hemoglobin (U. S. Patent 5,602,306, specifically incorporated herein by reference) and fibrinogen (U. S. Patent 5,639,940, ,  
15 specifically incorporated herein by reference) in non-human animals have also been disclosed, each specifically incorporated herein by reference in its entirety. Further, transgenic mice and rat models have recently been described as new directions to study and treat cardiovascular diseases such as hypertension in humans (Franz *et al.*, 1997; Pinto-Siestma and Paul, 1997). The construction of a transgenic mouse model has  
20 recently been used to assay potential treatments for Alzheimer's disease (U. S. Patent 5,720,936, specifically incorporated herein by reference in its entirety). It is contemplated in the present invention that transgenic animals contribute valuable information as models for studying the effects of ligands specific for the RXR nuclear hormone receptor in transgenic LXR $\alpha$  knockout animal models.

25

### **I. SELECTION AND SCREENING**

The present invention also provides recombinant candidate screening and selection methods which are based upon whole cell assays and which, preferably, employ a reporter gene that confers on its recombinant hosts a readily detectable phenotype that emerges only  
30 under conditions where a general DNA promoter positioned upstream of the reporter gene is



functional. Generally, reporter genes encode a polypeptide (marker protein) not otherwise produced by the host cell which is detectable by analysis of the cell culture, *e.g.*, by fluorometric, radioisotopic or spectrophotometric analysis of the cell culture.

5 Exemplary enzymes include esterases, phosphatases, proteases (tissue plasminogen activator or urokinase) and other enzymes capable of being detected by their activity, as will be known to those skilled in the art. More preferred for use in the present invention is green fluorescent protein (GFP) as a marker for transgene expression (Chalfie *et al.*, 1994). The use of GFP does not need exogenously added substrates, only irradiation by near UV or blue  
10 light, and thus has significant potential for use in monitoring gene expression in living cells. As the previously existing selection procedures for identifying correctly modified cells required culture of the manipulated cells for 10-14 days in a chemical known as G418, it was necessary to pass the cells to fresh feeders during the selection procedure. However, the use of the green fluorescent protein (GFP) as an identification marker allows for  
15 identification of transgenic colonies without the need for passage or addition of selectable media. As a results the cells remain healthier and, since are not passaged repeatedly, maintain their ability to generate a living offspring after nuclear transfer or blastocyst injection.

20 Other preferred examples are the enzyme chloramphenicol acetyltransferase (CAT) which may be employed with a radiolabelled substrate, firefly and bacterial luciferase, and the bacterial enzymes  $\beta$ -galactosidase and  $\beta$ -glucuronidase. Other marker genes within this class are well known to those of skill in the art, and are suitable for use in the present invention.

25 Another class of reporter genes which confer detectable characteristics on a host cell are those which encode polypeptides, generally enzymes, which render their transformants resistant against toxins. Examples of this class of reporter genes are the neo gene (Colberre-Garapin *et al.*, 1981) which protects host cells against toxic levels of the antibiotic G418,  
30 the gene conferring streptomycin resistance (U. S. Patent 4,430,434), the gene conferring

hygromycin B resistance (Santerre *et al.*, 1984; U. S. Patents 4,727,028, 4,960,704 and 4,559,302), a gene encoding dihydrofolate reductase, which confers resistance to methotrexate (Alt *et al.*, 1978), the enzyme HPRT, along with many others well known in the art (Kaufman, 1990).

5

The enzyme luciferase is useful as a screenable marker in the context of the present invention (Kang *et al.*, 1998). In the presence of the substrate luciferin, cells expressing luciferase emit light which can be detected on photographic or x-ray film, in a luminometer (or liquid scintillation counter), by devices that enhance night vision, or by a highly light sensitive video camera, such as a photon counting camera. All of these assays are nondestructive and transformed cells may be cultured further following identification. The photon counting camera is especially valuable as it allows one to identify specific cells or groups of cells which are expressing luciferase and manipulate those in real time. The above techniques also could be utilized if the screenable marker is the green fluorescent protein.

It is further contemplated that combinations of screenable and selectable markers will be useful for identification of transformed cells. In some cell or tissue types a selection agent, such as bialaphos or glyphosate, may either not provide enough killing activity to clearly recognize transformed cells or may cause substantial nonselective inhibition of transformants and nontransformants alike, thus causing the selection technique to not be effective. It is proposed that selection with a growth inhibiting compound, such as bialaphos or glyphosate at concentrations below those that cause 100% inhibition followed by screening of growing tissue for expression of a screenable marker gene such as luciferase would allow one to recover transformants from cell or tissue types that are not amenable to selection alone. Therefore it is proposed that combinations of selection and screening will enable one to identify transformants in a wider variety of cell and tissue types.

## II. CHARACTERIZATION

To confirm the presence of the exogenous DNA or "transgene(s)" in the transformed cells, a variety of assays may be performed. Such assays include, for example, "molecular biological" assays, such as Southern and Northern blotting, RT-PCR™ and PCR™; "biochemical" assays, such as detecting the presence of a protein product, *e.g.*, by immunological means (ELISAs and Western blots) or by enzymatic function assay.

## III. GENE EXPRESSION

While Southern blotting and PCR™ may be used to detect the LXRα gene(s) in question, they do not provide information as to whether the gene is being expressed. Expression may be evaluated by RT-PCR™ for mRNA and/or specifically identifying the protein products of the introduced genes or evaluating the phenotypic changes brought about by their expression.

Assays for the production and identification of specific proteins may make use of physical-chemical, structural, functional, or other properties of the proteins. Unique physical-chemical or structural properties allow the proteins to be separated and identified by electrophoretic procedures, such as native or denaturing gel electrophoresis or isoelectric focusing, or by chromatographic techniques such as ion exchange or gel exclusion chromatography. The unique structures of individual proteins offer opportunities for use of specific antibodies to detect their presence in formats such as an ELISA assay. Transgenic animals are described that synthesize epitope tagged prion proteins as a method of detecting the expressed protein(s) (U. S. Patent 5,789,655, specifically incorporated herein by reference in its entirety). Combinations of approaches may be employed with even greater specificity such as western blotting in which antibodies are used to locate individual gene products that have been separated by electrophoretic techniques. Additional techniques may be employed to absolutely confirm the identity of the product of interest such as evaluation by amino acid

sequencing following purification. Although these are among the most commonly employed, other procedures may be additionally used.

Assay procedures may also be used to identify the expression of proteins by their functionality, especially the ability of enzymes to catalyze specific chemical reactions involving specific substrates and products. These reactions may be followed by providing and quantifying the loss of substrates or the generation of products of the reactions by physical or chemical procedures. Examples are as varied as the enzyme to be analyzed and may include assays for PAT enzymatic activity by following production of radiolabeled acetylated phosphinothricin from phosphinothricin and  $^{14}\text{C}$ -acetyl CoA or for anthranilate synthase activity by following loss of fluorescence of anthranilate, to name two.

Very frequently the expression of a gene product is determined by evaluating the phenotypic results of its expression. These assays also may take many forms including but not limited to analyzing changes in the chemical composition, morphology, or physiological properties of the cells of the animal or human.

#### **IV. DNA INTEGRATION, RNA EXPRESSION AND INHERITANCE**

Genomic DNA may be isolated from animal cell lines or any animal parts to determine the presence of the exogenous gene through the use of techniques well known to those skilled in the art. Note, that intact sequences will not always be present, presumably due to rearrangement or deletion of sequences in the cell.

The presence of DNA elements introduced through the methods of this invention may be determined by polymerase chain reaction (PCR<sup>TM</sup>). Using this technique discreet fragments of DNA are amplified and detected by gel electrophoresis. This type of analysis permits one to determine whether a gene is present in a stable transformant, but does not prove integration of the introduced gene into the host cell genome. It is the experience of the inventors, however, that DNA has been integrated into the genome of

all transformants that demonstrate the presence of the gene through PCR™ analysis (Wu *et al.*, 1998). In addition, it is not possible using PCR™ techniques to determine whether transformants have exogenous genes introduced into different sites in the genome, *i.e.*, whether transformants are of independent origin. It is contemplated that using PCR™ techniques it would be possible to clone fragments of the host genomic DNA adjacent to an introduced gene.

Positive proof of DNA integration into the host genome and the independent identities of transformants may be determined using the technique of Southern hybridization. Using this technique specific DNA sequences that were introduced into the host genome and flanking host DNA sequences can be identified. Hence the Southern hybridization pattern of a given transformant serves as an identifying characteristic of that transformant. In addition it is possible through Southern hybridization to demonstrate the presence of introduced genes in high molecular weight DNA, *i.e.*, confirm that the introduced gene has been integrated into the host cell genome. The technique of Southern hybridization provides information that is obtained using PCR™ *e.g.*, the presence of a gene, but also demonstrates integration into the genome and characterizes each individual transformant.

It is contemplated that using the techniques of dot or slot blot hybridization which are modifications of Southern hybridization techniques one could obtain the same information that is derived from PCR™, *e.g.*, the presence of a gene.

Whereas DNA analysis techniques may be conducted using DNA isolated from any part of an animal, RNA will only be expressed in particular cells or tissue types and hence it will be necessary to prepare RNA for analysis from these tissues. PCR™ techniques may also be used for detection and quantitation of RNA produced from introduced genes. In this application of PCR™ it is first necessary to reverse transcribe RNA into DNA, using enzymes such as reverse transcriptase, and then through the use of conventional PCR™ techniques amplify the DNA. In most instances PCR™ techniques,

while useful, will not demonstrate integrity of the RNA product. Further information about the nature of the RNA product may be obtained by Northern blotting. This technique will demonstrate the presence of an RNA species and give information about the integrity of that RNA. The presence or absence of an RNA species can also be determined using dot or slot blot Northern hybridizations. These techniques are modifications of Northern blotting and will only demonstrate the presence or absence of an RNA species.

## **V. Methods of Transformation**

### **a. Electroporation**

In certain preferred embodiments of the present invention, the transgenic construct is introduced into the primordial germ cells via electroporation. Electroporation involves the exposure of a suspension of cells and DNA to a high-voltage electric discharge.

Transfection of eukaryotic cells using electroporation has been quite successful. Mouse pre-B lymphocytes have been transfected with human kappa-immunoglobulin genes (Potter *et al.*, 1984), and rat hepatocytes have been transfected with the chloramphenicol acetyltransferase gene (Tur-Kaspa *et al.*, 1986) in this manner.

It is contemplated that electroporation conditions for primordial germ cells from different sources may be optimized. One may particularly wish to optimize such parameters as the voltage, the capacitance, the time and the electroporation media composition. The execution of other routine adjustments will be known to those of skill in the art.

### **b. Particle Bombardment**

One of the preferred embodiments of the invention for transferring a naked DNA construct into cells involves particle bombardment. This method depends on the ability to accelerate DNA-coated microprojectiles to a high velocity allowing them to pierce cell membranes and enter cells without killing them (Klein *et al.*, 1987). The microprojectiles

used have consisted of biologically inert substances such as tungsten, platinum or gold beads.

It is contemplated that in some instances DNA precipitation onto metal particles would not be necessary for DNA delivery to a recipient cell using particle bombardment. It is contemplated that particles may contain DNA rather than be coated with DNA. Hence it is proposed that DNA-coated particles may increase the level of DNA delivery via particle bombardment but are not, in and of themselves, necessary.

Several devices for accelerating small particles have been developed. One such device relies on a high voltage discharge to generate an electrical current, which in turn provides the motive force (Yang *et al.*, 1990). Another method involves the use of a Biolistic Particle Delivery System, which can be used to propel particles coated with DNA through a screen, such as stainless steel or Nytex screen, onto a filter surface covered with cells in suspension. The screen disperses the particles so that they are not delivered to the recipient cells in large aggregates. It is believed that a screen intervening between the projectile apparatus and the cells to be bombarded reduces the size of projectile aggregates and may contribute to a higher frequency of transformation by reducing the damage inflicted on the recipient cells by projectiles that are too large.

For the bombardment, cells in suspension are preferably concentrated on filters, or alternatively on solid culture medium. The cells to be bombarded are positioned at an appropriate distance below the macroprojectile stopping plate. If desired, one or more screens are also positioned between the acceleration device and the cells to be bombarded.

In bombardment transformation, one may optimize the prebombardment culturing conditions and the bombardment parameters to yield the maximum numbers of stable transformants. Both the physical and biological parameters for bombardment are important in this technology. Physical factors are those that involve manipulating the

DNA/microprojectile precipitate or those that affect the flight and velocity or either the macro- or microprojectiles. Biological factors include all steps involved in manipulation of cells before and immediately after bombardment, the osmotic adjustment of target cells to help alleviate the trauma associated with bombardment, and also the nature of the transforming DNA, such as linearized DNA or intact supercoiled plasmids. It is believed that pre-bombardment manipulations are especially important for successful transformation of primordial germ cells.

Accordingly, it is contemplated that one may wish to adjust various of the bombardment parameters in small scale studies to fully optimize the conditions. One may particularly wish to adjust physical parameters such as gap distance, flight distance, tissue distance and helium pressure. One may also optimize the trauma reduction factors by modifying conditions which influence the physiological state of the recipient cells and which may therefore influence transformation and integration efficiencies. For example, the osmotic state, tissue hydration and the subculture stage or cell cycle of the recipient cells may be adjusted for optimum transformation. The execution of other routine adjustments will be known to those of skill in the art.

#### **c. Calcium Phosphate Co-Precipitation or DEAE-Dextran Treatment**

In other preferred embodiments of the present invention, the transgenic construct is introduced to the cells using calcium phosphate co-precipitation. Mouse primordial germ cells have been transfected with the SV40 large T antigen, with excellent results (Watanabe *et al.*, 1997). Human KB cells have been transfected with adenovirus 5 DNA (Graham and Van Der Eb, 1973) using this technique. Also in this manner, mouse L(A9), mouse C127, CHO, CV-1, BHK, NIH3T3 and HeLa cells were transfected with a neomycin marker gene (Chen and Okayama, 1987), and rat hepatocytes were transfected with a variety of marker genes (Rippe *et al.*, 1990).



In another embodiment, the expression construct is delivered into the cell using DEAE-dextran followed by polyethylene glycol. In this manner, reporter plasmids were introduced into mouse myeloma and erythroleukemia cells (Gopal, 1985).

5                    **d.        Direct Microinjection or Sonication Loading**

Further embodiments of the present invention include the introduction of the transgenic construct by direct microinjection or sonication loading. Direct microinjection has been used to introduce nucleic acid constructs into *Xenopus* oocytes (Harland and Weintraub, 1985), and LTK<sup>-</sup> fibroblasts have been transfected with the thymidine kinase  
10 gene by sonication loading (Fechheimer *et al.*, 1987).

**e.        Liposome Mediated Transformation**

In a further embodiment of the invention, the transgenic construct may be entrapped in a liposome. Liposomes are vesicular structures characterized by a  
15 phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat, 1991). Also  
20 contemplated is a transgenic construct complexed with Lipofectamine (Gibco BRL).

Liposome-mediated nucleic acid delivery and expression of foreign DNA *in vitro* has been very successful (Nicolau and Sene, 1982; Fraley *et al.*, 1979; Nicolau *et al.*, 1987). Wong *et al.* (1980) demonstrated the feasibility of liposome-mediated delivery  
25 and expression of foreign DNA in cultured chick embryo, HeLa and hepatoma cells.

In certain embodiments of the invention, the liposome may be complexed with a hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell membrane and promote cell entry of liposome-encapsulated DNA (Kaneda *et al.*, 1989).  
30 In other embodiments, the liposome may be complexed or employed in conjunction with

nuclear non-histone chromosomal proteins (HMG-1) (Kato *et al.*, 1991). In yet further embodiments, the liposome may be complexed or employed in conjunction with both HVJ and HMG-1.

5                                    **f.        Viral Transformation**

**i)        Retroviral Infection**

   The retroviruses are a group of single-stranded RNA viruses characterized by an ability to convert their RNA to double-stranded DNA in infected cells by a process of reverse-transcription (Coffin, 1990). The resulting DNA then stably integrates into  
10 cellular chromosomes as a provirus and directs synthesis of viral proteins. The integration results in the retention of the viral gene sequences in the recipient cell and its descendants. The retroviral genome contains three genes, gag, pol, and env that code for capsid proteins, polymerase enzyme, and envelope components, respectively. A sequence found upstream from the gag gene contains a signal for packaging of the genome into  
15 virions. Two long terminal repeat (LTR) sequences are present at the 5' and 3' ends of the viral genome. These contain strong promoter and enhancer sequences and are also required for integration in the host cell genome (Coffin, 1990).

   In order to construct a retroviral vector, a nucleic acid encoding a transgene of  
20 interest is inserted into the viral genome in the place of certain viral sequences to produce a virus that is replication-defective. In order to produce virions, a packaging cell line containing the gag, pol, and env genes but without the LTR and packaging components is constructed (Mann *et al.*, 1983). When a recombinant plasmid containing a cDNA, together with the retroviral LTR and packaging sequences is introduced into this cell line  
25 (by calcium phosphate precipitation for example), the packaging sequence allows the RNA transcript of the recombinant plasmid to be packaged into viral particles, which are then secreted into the culture media (Nicolas and Rubenstein, 1988; Temin, 1986; Mann *et al.*, 1983). The media containing the recombinant retroviruses is then collected, optionally concentrated, and used for gene transfer. Retroviral vectors are able to infect a

broad variety of cell types. However, integration and stable expression require the division of host cells (Paskind *et al.*, 1975).

Concern with the use of defective retrovirus vectors is the potential appearance of wild-type replication-competent virus in the packaging cells. This can result from recombination events in which the intact sequence from the recombinant virus inserts upstream from the gag, pol, env sequence integrated in the host cell genome. However, new packaging cell lines are now available that should greatly decrease the likelihood of recombination (Markowitz *et al.*, 1988; Hersdorffer *et al.*, 1990).

## ii) Adenoviral Infection

One method for delivery of the transgenic constructs involves the use of an adenovirus expression vector. Although adenovirus vectors are known to have a low capacity for integration into genomic DNA, this feature is counterbalanced by the high efficiency of gene transfer afforded by these vectors. "Adenovirus expression vector" is meant to include those constructs containing adenovirus sequences sufficient to (a) support packaging of the construct and (b) to ultimately express a transgenic construct that has been cloned therein.

The vector comprises a genetically engineered form of adenovirus. Knowledge of the genetic organization of adenovirus, a 36 kb, linear, double-stranded DNA virus, allows substitution of large pieces of adenoviral DNA with foreign sequences up to 7 kb (Grunhaus and Horwitz, 1992). In contrast to retrovirus, the adenoviral infection of host cells does not result in chromosomal integration because adenoviral DNA can replicate in an episomal manner without potential genotoxicity. Also, adenoviruses are structurally stable, and no genome rearrangement has been detected after extensive amplification.

Adenovirus is particularly suitable for use as a gene transfer vector because of its mid-sized genome, ease of manipulation, high titer, wide target-cell range and high infectivity. Both ends of the viral genome contain 100-200 base pair inverted repeats

(ITRs), which are *cis* elements necessary for viral DNA replication and packaging. The early (E) and late (L) regions of the genome contain different transcription units that are divided by the onset of viral DNA replication. The E1 region (E1A and E1B) encodes proteins responsible for the regulation of transcription of the viral genome and a few cellular genes. The expression of the E2 region (E2A and E2B) results in the synthesis of the proteins for viral DNA replication. These proteins are involved in DNA replication, late gene expression and host cell shut-off (Renan, 1990). The products of the late genes, including the majority of the viral capsid proteins, are expressed only after significant processing of a single primary transcript issued by the major late promoter (MLP). The MLP, (located at 16.8 m.u.) is particularly efficient during the late phase of infection, and all the mRNA's issued from this promoter possess a 5'-tripartite leader (TPL) sequence which makes them preferred mRNA's for translation.

In a current system, recombinant adenovirus is generated from homologous recombination between shuttle vector and provirus vector. Due to the possible recombination between two proviral vectors, wild-type adenovirus may be generated from this process. Therefore, it is critical to isolate a single clone of virus from an individual plaque and examine its genomic structure.

Generation and propagation of the current adenovirus vectors, which are replication deficient, depend on a unique helper cell line, designated 293, which was transformed from human embryonic kidney cells by Ad5 DNA fragments and constitutively expresses E1 proteins (Graham *et al.*, 1977). Since the E3 region is dispensable from the adenovirus genome (Jones and Shenk, 1978), the current adenovirus vectors, with the help of 293 cells, carry foreign DNA in either the E1, the D3 or both regions (Graham and Prevec, 1991). In nature, adenovirus can package approximately 105% of the wild-type genome (Ghosh-Choudhury *et al.*, 1987), providing capacity for about 2 extra kb of DNA. Combined with the approximately 5.5 kb of DNA that is replaceable in the E1 and E3 regions, the maximum capacity of the current adenovirus

vector is under 7.5 kb, or about 15% of the total length of the vector. More than 80% of the adenovirus viral genome remains in the vector backbone.

Helper cell lines may be derived from human cells such as human embryonic kidney cells, muscle cells, hematopoietic cells or other human embryonic mesenchymal or epithelial cells. Alternatively, the helper cells may be derived from the cells of other mammalian species that are permissive for human adenovirus. Such cells include, *e.g.*, Vero cells or other monkey embryonic mesenchymal or epithelial cells. As stated above, the preferred helper cell line is 293.

Recently, Racher *et al.* (1995) disclosed improved methods for culturing 293 cells and propagating adenovirus. In one format, natural cell aggregates are grown by inoculating individual cells into 1 liter siliconized spinner flasks (Techne, Cambridge, UK) containing 100-200 ml of medium. Following stirring at 40 rpm, the cell viability is estimated with trypan blue. In another format, Fibra-Cel microcarriers (Bibby Sterlin, Stone, UK) (5 g/l) is employed as follows. A cell inoculum, resuspended in 5 ml of medium, is added to the carrier (50 ml) in a 250 ml Erlenmeyer flask and left stationary, with occasional agitation, for 1 to 4 h. The medium is then replaced with 50 ml of fresh medium and shaking initiated. For virus production, cells are allowed to grow to about 80% confluence, after which time the medium is replaced (to 25% of the final volume) and adenovirus added at an MOI of 0.05. Cultures are left stationary overnight, following which the volume is increased to 100% and shaking commenced for another 72 h.

Other than the requirement that the adenovirus vector be replication defective, or at least conditionally defective, the nature of the adenovirus vector is not believed to be crucial to the successful practice of the invention. The adenovirus may be of any of the 42 different known serotypes or subgroups A-F. Adenovirus type 5 of subgroup C is the preferred starting material in order to obtain the conditional replication-defective adenovirus vector for use in the present invention. This is because Adenovirus type 5 is a human adenovirus about which a great deal of biochemical and genetic information is

known, and it has historically been used for most constructions employing adenovirus as a vector.

As stated above, the typical vector according to the present invention is replication defective and will not have an adenovirus E1 region. Thus, it will be most convenient to introduce the transforming construct at the position from which the E1-coding sequences have been removed. However, the position of insertion of the construct within the adenovirus sequences is not critical to the invention. The polynucleotide encoding the gene of interest may also be inserted in lieu of the deleted E3 region in E3 replacement vectors as described by Karlsson *et al.* (1986) or in the E4 region where a helper cell line or helper virus complements the E4 defect.

Adenovirus growth and manipulation is known to those of skill in the art, and exhibits broad host range *in vitro* and *in vivo*. This group of viruses can be obtained in high titers, *e.g.*,  $10^9$ - $10^{11}$  plaque-forming units per ml, and they are highly infective. The life cycle of adenovirus does not require integration into the host cell genome. The foreign genes delivered by adenovirus vectors are episomal and, therefore, have low genotoxicity to host cells. No side effects have been reported in studies of vaccination with wild-type adenovirus (Couch *et al.*, 1963; Top *et al.*, 1971), demonstrating their safety and therapeutic potential as *in vivo* gene transfer vectors.

Adenovirus vectors have been used in eukaryotic gene expression (Levrero *et al.*, 1991; Gomez-Foix *et al.*, 1992) and vaccine development (Grunhaus and Horwitz, 1992; Graham and Prevec, 1992). Recently, animal studies suggested that recombinant adenovirus could be used for gene therapy (Stratford-Perricaudet and Perricaudet, 1991; Stratford-Perricaudet *et al.*, 1990; Rich *et al.*, 1993). Studies in administering recombinant adenovirus to different tissues include trachea instillation (Rosenfeld *et al.*, 1991; Rosenfeld *et al.*, 1992), muscle injection (Ragot *et al.*, 1993), peripheral intravenous injections (Herz and Gerard, 1993) and stereotactic inoculation into the brain (Le Gal La Salle *et al.*, 1993).

### iii) AAV Infection

Adeno-associated virus (AAV) is an attractive vector system for use in the present invention as it has a high frequency of integration and it can infect nondividing cells, thus making it useful for delivery of genes into mammalian cells in tissue culture (Muzyczka, 1992). AAV has a broad host range for infectivity (Tratschin, *et al.*, 1984; Laughlin, *et al.*, 1986; Lebkowski, *et al.*, 1988; McLaughlin, *et al.*, 1988), which means it is applicable for use with the present invention. Details concerning the generation and use of rAAV vectors are described in U.S. Patent No. 5,139,941 and U.S. Patent No. 4,797,368, each incorporated herein by reference.

Studies demonstrating the use of AAV in gene delivery include LaFace *et al.* (1988); Zhou *et al.* (1993); Flotte *et al.* (1993); and Walsh *et al.* (1994). Recombinant AAV vectors have been used successfully for *in vitro* and *in vivo* transduction of marker genes (Kaplitt *et al.*, 1994; Lebkowski *et al.*, 1988; Samulski *et al.*, 1989; Shelling and Smith, 1994; Yoder *et al.*, 1994; Zhou *et al.*, 1994; Hermonat and Muzyczka, 1984; Tratschin *et al.*, 1985; McLaughlin *et al.*, 1988) and genes involved in human diseases (Flotte *et al.*, 1992; Luo *et al.*, 1994; Ohi *et al.*, 1990; Walsh *et al.*, 1994; Wei *et al.*, 1994). Recently, an AAV vector has been approved for phase I human trials for the treatment of cystic fibrosis.

AAV is a dependent parvovirus in that it requires coinfection with another virus (either adenovirus or a member of the herpes virus family) to undergo a productive infection in cultured cells (Muzyczka, 1992). In the absence of coinfection with helper virus, the wild type AAV genome integrates through its ends into human chromosome 19 where it resides in a latent state as a provirus (Kotin *et al.*, 1990; Samulski *et al.*, 1991). rAAV, however, is not restricted to chromosome 19 for integration unless the AAV Rep protein is also expressed (Shelling and Smith, 1994). When a cell carrying an AAV provirus is superinfected with a helper virus, the AAV genome is "rescued" from the chromosome or from a recombinant plasmid, and a normal productive infection is

established (Samulski *et al.*, 1989; McLaughlin *et al.*, 1988; Kotin *et al.*, 1990; Muzyczka, 1992).

Typically, recombinant AAV (rAAV) virus is made by cotransfecting a plasmid  
5 containing the gene of interest flanked by the two AAV terminal repeats (McLaughlin  
*et al.*, 1988; Samulski *et al.*, 1989; each incorporated herein by reference) and an  
expression plasmid containing the wild type AAV coding sequences without the terminal  
repeats, for example pIM45 (McCarty *et al.*, 1991; incorporated herein by reference).  
The cells are also infected or transfected with adenovirus or plasmids carrying the  
10 adenovirus genes required for AAV helper function. rAAV virus stocks made in such  
fashion are contaminated with adenovirus which must be physically separated from the  
rAAV particles (for example, by cesium chloride density centrifugation). Alternatively,  
adenovirus vectors containing the AAV coding regions or cell lines containing the AAV  
coding regions and some or all of the adenovirus helper genes could be used (Yang *et al.*,  
15 1994a; Clark *et al.*, 1995). Cell lines carrying the rAAV DNA as an integrated provirus  
can also be used (Flotte *et al.*, 1995).

#### iv) Other Viral Vectors

Other viral vectors may be employed as constructs in the present invention.  
20 Vectors derived from viruses such as vaccinia virus (Ridgeway, 1988; Baichwal and  
Sugden, 1986; Coupar *et al.*, 1988) and herpesviruses may be employed. They offer  
several attractive features for various mammalian cells (Friedmann, 1989; Ridgeway,  
1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988; Horwich *et al.*, 1990).

25 With the recent recognition of defective hepatitis B viruses, new insight was  
gained into the structure-function relationship of different viral sequences. *In vitro*  
studies showed that the virus could retain the ability for helper-dependent packaging and  
reverse transcription despite the deletion of up to 80% of its genome (Horwich *et al.*,  
1990). This suggested that large portions of the genome could be replaced with foreign  
30 genetic material. Chang *et al.* recently introduced the chloramphenicol acetyltransferase



(CAT) gene into duck hepatitis B virus genome in the place of the polymerase, surface, and pre-surface coding sequences. It was cotransfected with wild-type virus into an avian hepatoma cell line. Culture media containing high titers of the recombinant virus were used to infect primary duckling hepatocytes. Stable CAT gene expression was detected for at least 24 days after transfection (Chang *et al.*, 1991).

In still further embodiments of the present invention, the nucleic acids to be delivered are housed within an infective virus that has been engineered to express a specific binding ligand. The virus particle will thus bind specifically to the cognate receptors of the target cell and deliver the contents to the cell. A novel approach designed to allow specific targeting of retrovirus vectors was recently developed based on the chemical modification of a retrovirus by the chemical addition of lactose residues to the viral envelope. This modification can permit the specific infection of hepatocytes via sialoglycoprotein receptors.

Another approach to targeting of recombinant retroviruses was designed in which biotinylated antibodies against a retroviral envelope protein and against a specific cell receptor were used. The antibodies were coupled via the biotin components by using streptavidin (Roux *et al.*, 1989). Using antibodies against major histocompatibility complex class I and class II antigens, they demonstrated the infection of a variety of human cells that bore those surface antigens with an ecotropic virus *in vitro* (Roux *et al.*, 1989).

#### **g. Adenoviral Assisted Transfection**

In certain embodiments of the present invention, the transgenic construct is introduced into the cell using adenovirus assisted transfection. Increased transfection efficiencies have been reported in cell systems using adenovirus coupled systems (Kelleher and Vos, 1994; Cotten *et al.*, 1992; Curiel, 1994), and the inventors contemplate using the same technique to increase transfection efficiencies.

#### h. Receptor Mediated Transfection

Still further constructs that may be employed to deliver the transgenic construct to the target cells are receptor-mediated delivery vehicles. These take advantage of the selective uptake of macromolecules by receptor-mediated endocytosis that will be occurring in the target cells. In view of the cell type-specific distribution of various receptors, this delivery method adds a degree of specificity to the present invention. Specific delivery in the context of another mammalian cell type is described by Wu and Wu (1993; incorporated herein by reference).

Certain transgenic delivery constructs comprise a cell receptor-specific ligand and a DNA-binding agent. Others comprise a cell receptor-specific ligand to which the DNA construct to be delivered has been operatively attached. Several ligands have been used for receptor-mediated gene transfer (Wu and Wu, 1987; Wagner *et al.*, 1990; Ferkol *et al.*, 1993; Perales *et al.*, 1994; Myers, EPO 0273085), which establishes the operability of the technique.

In other embodiments, the DNA delivery vehicle component may comprise a specific binding ligand in combination with a liposome. The nucleic acids to be delivered are housed within the liposome and the specific binding ligand is functionally incorporated into the liposome membrane. The liposome will thus specifically bind to the receptors of the target cell and deliver the contents to the cell. Such systems have been shown to be functional using systems in which, for example, epidermal growth factor (EGF) is used in the receptor-mediated delivery of a nucleic acid to cells that exhibit upregulation of the EGF receptor.

In still further embodiments, the DNA delivery vehicle component of the delivery vehicles may be a liposome itself, which will preferably comprise one or more lipids or glycoproteins that direct cell-specific binding. For example, Nicolau *et al.* (1987) employed lactosyl-ceramide, a galactose-terminal asialoganglioside, incorporated into liposomes and observed an increase in the uptake of the insulin gene by hepatocytes. It is

contemplated that the transgenic constructs of the present invention can be specifically delivered into the target cells in a similar manner.

## VI. VECTORS AND REGULATORY SIGNALS

5 Vectors of the present invention are designed, primarily, to produce non-functional alleles in LXR $\alpha$ . As described above in the section dealing with transgenic animals, creation of non-functional alleles can result from a variety of different genetic changes. These include interruption of the gene, deletion of the gene, introduction of a stop codon, or alteration of a regulatory signal. Other methods include alteration of a  
10 splice site or some signal required for other post-transcriptional or post-translational processing.

In many instances, the vectors of the present invention do not encode a functional LXR $\alpha$  gene and, thus, need not contain a promoter driving that gene. However, it may  
15 prove useful to provide functional LXR $\alpha$  genes with regulatable promoters (*i.e.*, inducible, repressable, tissue specific). Also, the vectors usually will contain a selectable marker if, for no other reason, to facilitate their production *in vitro*. However, selectable markers may play an important role in producing transgenic cells and animals. Thus, a discussion of promoters is useful here.

20

**Table 1 - Inducible Elements**

Element	Inducer	References
MT II	Phorbol Ester (TFA) Heavy metals	Palmiter <i>et al.</i> , 1982; Haslinger and Karin, 1985; Searle <i>et al.</i> , 1985; Stuart <i>et al.</i> , 1985; Imagawa <i>et al.</i> , 1987; Karin ®, 1987; Angel <i>et al.</i> , 1987b; McNeall <i>et al.</i> , 1989
MMTV (mouse mammary tumor virus)	Glucocorticoids	Huang <i>et al.</i> , 1981; Lee <i>et al.</i> , 1981; Majors and Varmus, 1983; Chandler <i>et al.</i> , 1983; Lee <i>et al.</i> , 1984; Fonta <i>et al.</i> , 1985; Sakai <i>et al.</i> , 1986

Element	Inducer	References
$\beta$ -Interferon	poly(rI)X poly(rc)	Tavernier <i>et al.</i> , 1983
Adenovirus 5 E2	Ela	Imperiale and Nevins, 1984
Collagenase	Phorbol Ester (TPA)	Angle <i>et al.</i> , 1987a
Stromelysin	Phorbol Ester (TPA)	Angle <i>et al.</i> , 1987b
SV40	Phorbol Ester (TFA)	Angel <i>et al.</i> , 1987b
Murine MX Gene	Interferon, Newcastle Disease Virus	
GRP78 Gene	A23187	Resendez <i>et al.</i> , 1988
$\alpha$ -2-Macroglobulin	IL-6	Kunz <i>et al.</i> , 1989
Vimentin	Serum	Rittling <i>et al.</i> , 1989
MHC Class I Gene H-2kb	Interferon	Blonar <i>et al.</i> , 1989
HSP70	Ela, SV40 Large T Antigen	Taylor <i>et al.</i> , 1989; Taylor and Kingston, 1990a,b
Proliferin	Phorbol Ester-TPA	Mordacq and Linzer, 1989
Tumor Necrosis Factor	FMA	Hensel <i>et al.</i> , 1989
Thyroid Stimulating Hormone $\alpha$ Gene	Thyroid Hormone	Chatterjee <i>et al.</i> , 1989

**Table 2 - Other Promoter/Enhancer Elements**

Promoter/Enhancer	References
Immunoglobulin Heavy Chain	Hanerji <i>et al.</i> , 1983; Gilles <i>et al.</i> , 1983; Grosschedl and Baltimore, 1985; Atchinson and Perry, 1986, 1987; Imler <i>et al.</i> , 1987; Weinberger <i>et al.</i> , 1988; Kiledjian <i>et al.</i> , 1988; Porton <i>et al.</i> , 1990
Immunoglobulin Light Chain	Queen and Baltimore, 1983; Picard and Schaffner, 1984
T-Cell Receptor	Luria <i>et al.</i> , 1987, Winoto and Baltimore, 1989; Redondo <i>et al.</i> , 1990
HLA DQ $\alpha$ and DQ $\beta$	Sullivan and Peterlin, 1987
$\beta$ -Interferon	Goodbourn <i>et al.</i> , 1986; Fujita <i>et al.</i> , 1987; Goodbourn and Maniatis, 1985

Promoter/Enhancer	References
Interleukin-2	Greene <i>et al.</i> , 1989
Interleukin-2 Receptor	Greene <i>et al.</i> , 1989; Lin <i>et al.</i> , 1990
MHC Class II 5	Koch <i>et al.</i> , 1989
MHC Class II HLA-DR $\alpha$	Sherman <i>et al.</i> , 1989
$\beta$ -Actin	Kawamoto <i>et al.</i> , 1988; Ng <i>et al.</i> , 1989
Muscle Creatine Kinase	Jaynes <i>et al.</i> , 1988; Horlick and Benfield, 1989; Johnson <i>et al.</i> , 1989a
Prealbumin (Transthyretin)	Costa <i>et al.</i> , 1988
Elastase I	Omitz <i>et al.</i> , 1987
Metallothionein	Karin <i>et al.</i> , 1987; Culotta and Hamer, 1989
Collagenase	Pinkert <i>et al.</i> , 1987; Angel <i>et al.</i> , 1987
Albumin Gene	Pinkert <i>et al.</i> , 1987, Tronche <i>et al.</i> , 1989, 1990
$\alpha$ -Fetoprotein	Godbout <i>et al.</i> , 1988; Campere and Tilghman, 1989
t-Globin	Bodine and Ley, 1987; Perez-Stable and Constantini, 1990
$\beta$ -Globin	Trudel and Constantini, 1987
e-fos	Cohen <i>et al.</i> , 1987
c-HA-ras	Triesman, 1986; Deschamps <i>et al.</i> , 1985
Insulin	Edlund <i>et al.</i> , 1985
Neural Cell Adhesion Molecule (NCAM)	Hirsch <i>et al.</i> , 1990
$\alpha$ 1-Antitrypsin	Latimer <i>et al.</i> , 1990
H2B (TH2B) Histone	Hwang <i>et al.</i> , 1990
Mouse or Type I Collagen	Ripe <i>et al.</i> , 1989
Glucose-Regulated Proteins (GRP94 and GRP78)	Chang <i>et al.</i> , 1989
Rat Growth Hormone	Larsen <i>et al.</i> , 1986
Human Serum Amyloid A (SAA)	Edbrooke <i>et al.</i> , 1989

Promoter/Enhancer	References
Troponin I (TN I)	Yutzey <i>et al.</i> , 1989
Platelet-Derived Growth Factor	Pech <i>et al.</i> , 1989
Duchenne Muscular Dystrophy	Klamut <i>et al.</i> , 1990
SV40	Banerji <i>et al.</i> , 1981; Moreau <i>et al.</i> , 1981; Sleight and Lockett, 1985; Firak and Subramanian, 1986; Herr and Clarke, 1986; Imbra and Karin, 1986; Kadesch and Berg, 1986; Wang and Calame, 1986; Ondek <i>et al.</i> , 1987; Kuhl <i>et al.</i> , 1987 Schaffner <i>et al.</i> , 1988
Polyoma	Swartzendruber and Lehman, 1975; Vasseur <i>et al.</i> , 1980; Katinka <i>et al.</i> , 1980, 1981; Tyndell <i>et al.</i> , 1981; Dandolo <i>et al.</i> , 1983; deVilliers <i>et al.</i> , 1984; Hen <i>et al.</i> , 1986; Satake <i>et al.</i> , 1988; Campbell and Villarreal, 1988
Retroviruses	Kriegler and Botchan, 1982, 1983; Levinson <i>et al.</i> , 1982; Kriegler <i>et al.</i> , 1983, 1984a,b, 1988; Bosze <i>et al.</i> , 1986; Miksicek <i>et al.</i> , 1986; Celander and Haseltine, 1987; Thiesen <i>et al.</i> , 1988; Celander <i>et al.</i> , 1988; Chol <i>et al.</i> , 1988; Reisman and Rotter, 1989
Papilloma Virus	Campo <i>et al.</i> , 1983; Lusky <i>et al.</i> , 1983; Spandidos and Wilkie, 1983; Spalholz <i>et al.</i> , 1985; Lusky and Botchan, 1986; Cripe <i>et al.</i> , 1987; Gloss <i>et al.</i> , 1987; Hirochika <i>et al.</i> , 1987, Stephens and Hentschel, 1987; Glue <i>et al.</i> , 1988
Hepatitis B Virus	Bulla and Siddiqui, 1986; Jameel and Siddiqui, 1986; Shaul and Ben-Levy, 1987; Spandau and Lee, 1988; Vannice and Levinson, 1988
Human Immunodeficiency Virus	Muesing <i>et al.</i> , 1987; Hauber and Cullan, 1988; Jakobovits <i>et al.</i> , 1988; Feng and Holland, 1988; Takebe <i>et al.</i> , 1988; Rowen <i>et al.</i> , 1988; Berkhout <i>et al.</i> , 1989; Laspia <i>et al.</i> , 1989; Sharp and Marciniak, 1989; Braddock <i>et al.</i> , 1989
Cytomegalovirus	Weber <i>et al.</i> , 1984; Boshart <i>et al.</i> , 1985; Foecking and Hofstetter, 1986
Gibbon Ape Leukemia Virus	Holbrook <i>et al.</i> , 1987; Quinn <i>et al.</i> , 1989

Another signal that may prove useful are polyadenylation signals (hGH, BGH, SV40).

In certain embodiments of the invention, the use of internal ribosome binding sites (IRES) elements are used to create multigene, or polycistronic, messages. IRES elements are able to bypass the ribosome scanning model of 5'-methylated cap-dependent translation and begin translation at internal sites (Pelletier and Sonenberg, 1988). IRES elements from two members of the picornavirus family (polio and encephalomyocarditis) have been described (Pelletier and Sonenberg, 1988), as well an IRES from a mammalian message (Macejak and Sarnow, 1991). IRES elements can be linked to heterologous open reading frames. Multiple open reading frames can be transcribed together, each separated by an IRES, creating polycistronic messages. By virtue of the IRES element, each open reading frame is accessible to ribosomes for efficient translation. Multiple genes can be efficiently expressed using a single promoter/enhancer to transcribe a single message.

As discussed above, in certain embodiments of the invention, a cell may be identified and selected *in vitro* or *in vivo* by including a marker in the expression construct. Such markers confer an identifiable change to the cell permitting easy identification of cells containing the expression construct. Usually, the inclusion of a drug selection marker aids in cloning and in the selection of transformants, for example, genes that confer resistance to neomycin, puromycin, hygromycin, DHFR, GPT, zeocin, tetracycline and histidinol are useful selectable markers. Alternatively, enzymes such as herpes simplex virus thymidine kinase (*tk*) or chloramphenicol acetyltransferase (CAT) may be employed.

The promoters and enhancers that control the transcription of protein encoding genes in eukaryotic cells are composed of multiple genetic elements. The cellular machinery is able to gather and integrate the regulatory information conveyed by each

element, allowing different genes to evolve distinct, often complex patterns of transcriptional regulation.

5 The term promoter will be used here to refer to a group of transcriptional control modules that are clustered around the initiation site for RNA polymerase II. Much of the thinking about how promoters are organized derives from analyses of several viral promoters, including those for the HSV thymidine kinase (tk) and SV40 early transcription units. These studies, augmented by more recent work, have shown that promoters are composed of discrete functional modules, each consisting of approximately 10 7-20 bp of DNA, and containing one or more recognition sites for transcriptional activator proteins.

At least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters 15 lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV 40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation. 20 Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between elements is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between elements can be increased to 50 bp apart 25 before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

Enhancers were originally detected as genetic elements that increased transcription from a promoter located at a distant position on the same molecule of DNA. 30 This ability to act over a large distance had little precedent in classic studies of



prokaryotic transcriptional regulation. Subsequent work showed that regions of DNA with enhancer activity are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins.

5           The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities.  
10       Aside from this operational distinction, enhancers and promoters are very similar entities.

          Promoters and enhancers have the same general function of activating transcription in the cell. They are often overlapping and contiguous, often seeming to have a very similar modular organization. Taken together, these considerations suggest  
15       that enhancers and promoters are homologous entities and that the transcriptional activator proteins bound to these sequences may interact with the cellular transcriptional machinery in fundamentally the same way.

          In any event, it will be understood that promoters are DNA elements which when  
20       positioned functionally upstream of a gene leads to the expression of that gene. Most transgene constructs of the present invention are functionally positioned downstream of a promoter element.

**a.       Promoters Specific for Undifferentiated Cells**

25       Preferred for use in the present invention are promoters which are active in undifferentiated cells. Promoters that lead to high levels of expression in undifferentiated cells include the phosphoglycerate kinase (pgk) promoter and the octamer binding transcription factor 4 (Oct-4) promoter. The pgk promoter is known to lead to high levels of expression in undifferentiated mouse ES cells.

30

Transgenic experiments have identified a regulatory region upstream of the Oct-4 gene that is capable of targeting high level expression to the undifferentiated inner cell mass and the primordial germ cells (Yeom *et al.*, 1996). By using the Oct-4 promoter, expression would be limited to highly undifferentiated cells and PGCs, thereby allowing  
5 for selection of the cells with the highest probability of contributing to the germ line by measurement of the expression levels of a selected marker protein, preferably green fluorescent protein. Use of the Oct-4 promoter also allows for early screening of putative transgenic animals, saving considerable time and expense, by not requiring that chimeras be saved until breeding age for testing, and by not requiring expensive and prolonged  
10 breeding tests.

#### **b. Eukaryotic and Viral Promoters and Enhancers**

Preferred for use in the present invention is the cytomegalovirus (CMV) promoter. This promoter is commercially available from Invitrogen in the vector pcDNAIII, which  
15 is preferred for use in the present invention. Below are a list of additional viral promoters, cellular promoters/enhancers and inducible promoters/enhancers that could be used in combination with the present invention. Additionally any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression of structural genes encoding oligosaccharide processing enzymes,  
20 protein folding accessory proteins, selectable marker proteins or a heterologous protein of interest.

#### **E. Screening Assays**

The present invention provides methods of screening compounds for a variety of  
25 different activities using a number different assay formats. The assays, at their most basic level, assess the impact that a given candidate substance will have upon a cell, tissue, organ or organism, as compared to that same cell, tissue organ or organism in the absence of the candidate substance. In certain embodiments, using genetically related but distinct cells, it is possible to ascertain some information about how the candidate substance is  
30 affecting the target.

In some instances, for example, where cholesterol levels or adsorption are to be measured, it may prove useful to use a system where there is increased cholesterol introduced into the test system, be that system a cell, a cell culture, a tissue, and organ or an organism. Such "high cholesterol" media or diets are well known to those of skill in the art. As an example, a 2% cholesterol diet is utilized for testing in mice and designated "high cholesterol" for the purposes of this study.

As used herein, the term "candidate substance" refers to any molecule. The candidate substance may be a classic pharmaceutical, a peptide, a protein or fragment thereof, a synthetic small molecule, or an oligo- or polynucleotide or a lipid. The active compounds also include naturally-occurring substances, fragments or parts thereof, for example, compounds isolated from natural sources, such as animals, bacteria, fungi, plant sources, including leaves and bark, and marine samples. Also included are active combinations of known compounds which are inactive or suboptimally active when administered separately.

It may prove to be the case that the most useful compounds for identification through application of a screening assay will be compounds that are structurally related to known compounds that are found to have desired activities. So-called "rational drug design," which is based on the structural or physical attributes of a given candidate substance and its similarity or difference with respect to functional and non-functional substances, permits a directed method of modification towards the goal of improving or optimizing function. Where a particular molecular target is known, rational drug design may also take into account one or more structural or physical attributes of the target when designing or modifying the candidate substances.

Screening assays are relatively simple to set up and perform. Thus, in assaying for a candidate substance, one must provide a suitable system in which to test the substance. Typically, the system is a cell or cell culture, or an organism. One then either contacts the

candidate substance with the cell, or administers the candidate substance to the organism. One then measures some read out of cellular, tissue, organ or organismal function or characteristic to ascertain the effect of the candidate substance. Of course, this read out takes into account, or actually measures, the cellular, tissue, organ or organismal function or characteristic in an untreated cell, tissue, organ or organism.

It also must be recognized that some substances may not prove to have an effect at lower dosages ranges. Therefore, it may prove useful to conduct dose response experiments, especially when there is no related compound upon which to base an estimate of the effective amount. However, solubility and toxicity considerations usually are available to provide a reasonable upper limit upon the amount of a substance that will be tested.

Two particular classes of compounds will hold particular significance in the following methods -- RXR agonists and LXR $\alpha$  agonists. As discussed above, these two molecules form a heterodimer which is an important receptor for regulating, among other things, bile acid synthesis. The natural agonist of RXR is 9-cis retinoic acid. One particular class of RXR agonists is the rexinoids, synthetic analogs of retinoic acids. Various rexinoids are described in U.S. Patents 5,780,676 and 5,455,265, which are incorporated by reference. The present inventors have shown that some, but not all, rexinoid agonists of RXR are capable of decreasing cholesterol uptake.

The other class of compounds that will be of particular interest are agonists of LXR $\alpha$ . The natural agonists for LXR $\alpha$  are oxysterols, oxygenated metabolic derivatives of cholesterol. Selected analogs of these compounds should prove particularly useful in increasing bile acid synthesis.

As discussed below, there are a variety of different assays, and hence, a variety of different read outs for "activity." The examples provided below should not be considered limiting and are merely exemplary. The skilled artisan is well aware of other examples of

read outs depending on the nature of the assay and the intent of the screen. It will, of course, be understood that all the screening methods of the present invention are useful in themselves notwithstanding the fact that effective candidates may not be found. The invention provides methods for screening for such candidates, not solely methods of finding them.

Also, while the specific embodiments described below focus on *in vitro* and *in vivo* examples, the present inventors also contemplate *ex vivo* experiments using whole tissues or organs derived from suitable research animals, human tissue donors or cadavers.

## **I. METHOD FOR SCREENING FOR INCREASE IN BILE ACID SYNTHESIS**

### **a. *In vitro***

Bile acids are an important tool in metabolizing cholesterol and, thus, may provide one mechanism by which abnormally high cholesterol levels may be countered *in vivo*. Therefore, one screening assay will focus on the ability of candidate substances to increase bile acid synthesis. This method generally includes the steps of:

- (a) providing a cell;
- (b) contacting said cell with said candidate substance; and
- (c) monitoring a bile acid-related phenotype, as compared to a similar cell not treated with said candidate substance.

If there is an increase in the bile acid-related phenotype in the treated, as compared to the untreated cell, the candidate substance is identified as having the ability to increase bile acid synthesis. In this particular embodiment, the compounds to be tested are RXR and LXR $\alpha$  agonists. With respect to the former, one particular group of interest are the rexinoid compounds (see above).

5 The cell of interest should contain RXR/LXR $\alpha$  receptors in sufficient amounts to effect a measurable change in the phenotype of the cell. Cells particularly suited to this purpose are liver cells, and may be subjected to a high cholesterol environment. Use of primary hepatocytes is a preferred embodiment. One may measure any bile-acid related phenotype, but a particular phenotype is the induction of cholesterol 7 $\alpha$ -hydroxylase (Cyp7a) expression. This gene is the rate limiting enzyme in bile acid synthesis.

10 Though a normal cell can be utilized in this assay, the ability to discern the bile acid induced versus uninduced states of such cells may be difficult. For example, in a normal cholesterol environment, there will be little stimulation of bile acid production. Induction with a candidate substance may, in and of itself, result in a fairly significant increase in bile acid production. Alternatively, the absence of oxysterol may handicap the cell such that induction is not possible. In a high cholesterol environment, however, oxysterol will be plentiful and bile acid synthesis will be activated. In this case, however, it may be difficult to observe any "superinduction" by the candidate substance.

15 A convenient compromise between these two situations is where the cell carries only a single functional LXR $\alpha$  allele. In this case, the high cholesterol environment, which should enable induction of bile acid synthesis, will not "max out" bile acid synthesis because only about one-half of the receptor capacity of the cells is being expressed. It is important that these cells still retain one functional LXR $\alpha$  allele, since loss of both alleles would render the cells completely unresponsive to RXR/LXR $\alpha$  agonists. Thus, cells that are genetically altered to be heterozygous for the LXR $\alpha$  allele 25 (+/-) will prove particularly useful.

#### **b. *In vivo***

30 As a parallel to the preceding assay, one may wish to examine effects of a candidate substance on bile acid synthesis *in vivo*. This assay will be similar to the preceding *in vitro* assay with the following notable exceptions. First, obviously, the

candidate substance is provided to an animal rather than a cell or cell culture. Second, the readouts in the assay will include cholesterol levels, Cyp7a synthesis, fecal bile acid excretion, bile acid pool size and bile acid composition. Third, one may wish to screen more than just RXR and LXR $\alpha$  agonists for increases in bile acid synthesis. In this case, one will utilize a heterozygous LXR $\alpha$  (+/-) knockout animal.

## **II. METHOD FOR SCREENING FOR DECREASE IN CHOLESTEROL ADSORPTION**

### **a. *In vitro***

In another embodiment, the present invention provides for the screening of rexinoid compounds for their ability to inhibit adsorption of cholesterol by intestinal cells. A high cholesterol environment can be utilized. The method includes the steps of:

- (a) providing an intestinal cell;
- (b) contacting said cell with the candidate rexinoid; and
- (c) monitoring cholesterol absorption by said cell, as compared to a similar cell not treated with said rexinoid.

A decrease in absorption of cholesterol in the treated cell, as compared to the untreated cell, indicates that the rexinoid is an inhibitor of cholesterol absorption. Monitoring of uptake of a labeled (radioactive, chemilluminescent, fluorometric) cholesterol is one method of measuring activity. A particularly preferred target cell is a duodenal cell.

As mentioned briefly, above, it may prove useful to examine the effects of an inhibitor, identified according to this method, on cells comprising one or two non-functional LXR $\alpha$  alleles. By showing the dependence of the inhibitor on the presence of a functional RXR/LXR $\alpha$ , information can be gained about how the inhibitor is effecting a decrease in cholesterol absorption.

It also may prove useful, in both the *in vivo* and *in vitro* embodiments, to add another an agent that inhibits cholesterol biosynthesis. Together, these agents may prove to act even better in concert than they do individually. A group of compounds that are used to inhibit cholesterol biosynthesis are HMG CoA reductase inhibitors, the so-called “Statins.”

**b. *In vivo***

Again, it may prove useful to screen for effects of a potential inhibitor of cholesterol absorption *in vivo*. This assay will be similar to the preceding *in vitro* assay with the following notable exceptions. First, obviously, the candidate substance is provided to an animal rather than a cell or cell culture. Second, the readouts in the assay will include cholesterol absorption directly, or indirectly by measuring circulating cholesterol levels, hepatic cholesterol, hepatomegaly, atherosclerosis, cardiac failure, cardiac hypertrophy or atrophy, activity level, survival, cancer, reproduction, immune function, skin disease, cognitive function and adrenal function. Particularly preferred will be the assay reported by Turley *et al.* (1994), which is incorporated by reference. Third, one may wish to screen more than just rexinoids for inhibition of cholesterol absorption. In this case, one will utilize a homozygous heterozygous LXR $\alpha$  (-/-; +/-) knockout animal.

**III. METHOD FOR SCREENING FOR REDUCTION IN *IN VIVO* CHOLESTEROL LEVELS**

In another method, one may wish to look directly at the effects of a compound, or a combination of compounds, on cholesterol levels *in vivo*. The method includes the steps of:

- (a) providing a non-human transgenic mammal, the cells of which comprise at least one non-functional endogenous LXR $\alpha$  allele;
- (b) treating said mammal with a candidate substance; and



- (c) monitoring a cholesterol-related phenotype in said mammal, as compared to a similar mammal not treated with said candidate substance.

5 A change in the cholesterol phenotype indicative of a decrease in cholesterol levels in the treated cell, as compared to the untreated cell, indicates that the candidate substance is an inhibitor of cholesterol levels. Again, one may wish to use other compounds in combination, be they of known or unknown activity on cholesterol levels, in the same assay. The readouts in the assay will include cholesterol absorption directly, or indirectly by measuring circulating cholesterol levels, hepatic cholesterol, hepatomegaly, 10 atherosclerosis, cardiac failure, cardiac hypertrophy or atrophy, activity level, survival, cancer, reproduction, immune function, skin disease, cognitive function and adrenal function. Preferably, the mammal is subjected to a high cholesterol diet.

15 One could also screen specifically for an RXR (*e.g.*, rexinoid) or LXR agonist's effect on cholesterol levels using a normal animal. This embodiment would likely contemplate only the use of high cholesterol diet.

#### IV. METHOD FOR SCREENING FOR MODULATION OF ABC1 EXPRESSION

20 In another method, one may wish to look directly at the effects of a compound, or a combination of compounds, on ABC-1 levels *in vitro* or *in vivo*. The method includes the steps of screening for a modulator of ABC1 expression comprising:

- (a) providing a cell expressing an RXR;
- (b) contacting said cell with a rexinoid and a candidate substance;
- 25 (c) and determining the expression of ABC1 in said cell,
- wherein a change in expression of ABC1, as compared to a cell of step (b), indicates that said candidate substance is a modulator of ABC1 expression.

30 Screening for a modulator of ABC1 expression further comprises the step of determining the expression of ABC1 in a cell expressing RXR in the absence of said

candidate substance. ABC1 expression is measured by RNA analysis, such as Northern analysis or PCR. Alternatively, ABC1 expression is measured by protein analysis, such as ELISA or Western blot. In preferred embodiments, screening for a modulator of ABC1 expression in a cell comprises an exogenous marker cassette comprising a polynucleotide encoding a screenable marker operably linked to an ABC1 promoter region. The screenable marker may be an esterase, phosphatase, protease, green fluorescent protein, luciferase, chloramphenicol acetyl transferase,  $\beta$ -galactosidase,  $\beta$ -glucuronidase or a drug resistance marker. Preferred cells for screening for a modulator of ABC1 expression are cells expressing an RXR such as intestinal cells, preferably of duodenal or jejunal origin. Screening for a modulator of ABC1 expression may be done *in vivo*.

The present invention also provides for a method of making a modulator of ABC1 expression comprising: 1) providing a cell expressing an RXR; 2) contacting said cell with a rexinoid and a candidate substance; 3) determining the expression of ABC1 in said cell, wherein a change in expression of ABC1, as compared to a cell of step (b), indicates that said candidate substance is a modulator of ABC1 expression; and 4) making said modulator.

## **F. Pharmaceutical Compositions and Routes of Delivery**

In certain embodiments, the present invention also concerns formulations of one or more agonist compositions for administration to a mammal, that inhibits cholesterol biosynthesis or cholesterol absorption. It will also be understood that, if desired, the agonist compositions disclosed herein may be administered in combination with other agents as well, such as, *e.g.*, various pharmaceutically-active agents. As long as the composition comprises at least one cholesterol inhibitory agonist, there is virtually no limit to other components which may also be included, given that the additional agents do not cause a significant adverse effect upon contact with the target cells or host tissues.

5 The formulation of pharmaceutically-acceptable excipients and carrier solutions are well-known to those of skill in the art, as is the development of suitable dosing and treatment regimens for using the particular compositions described herein in a variety of treatment regimens, including *e.g.*, oral, parenteral, intravenous, intranasal, and intramuscular administration and formulation.

### **I. ORAL COMPOSITIONS AND DELIVERY**

10 The pharmaceutical compositions disclosed herein may be delivered *via* oral administration to an animal, and as such, these compositions may be formulated with an inert diluent or with an assimilable edible carrier, or they may be enclosed in hard- or soft-shell gelatin capsule, or they may be compressed into tablets, or they may be incorporated directly with the food of the diet.

15 The active compounds may even be incorporated with excipients and used in the form of ingestible tablets, buccal tables, troches, capsules, elixirs, suspensions, syrups, wafers, and the like (Mathiowitz *et al.*, 1997; Hwang *et al.*, 1998; U. S. Patent 5,641,515; U. S. Patent 5,580,579 and U. S. Patent 5,792,451, each specifically incorporated herein by reference in its entirety). The tablets, troches, pills, capsules and the like may also contain the following: a binder, as gum tragacanth, acacia, cornstarch, or gelatin; 20 excipients, such as dicalcium phosphate; a disintegrating agent, such as corn starch, potato starch, alginic acid and the like; a lubricant, such as magnesium stearate; and a sweetening agent, such as sucrose, lactose or saccharin may be added or a flavoring agent, such as peppermint, oil of wintergreen, or cherry flavoring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. 25 Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compounds sucrose as a sweetening agent methyl and propylparabens as preservatives, a dye and flavoring, such as cherry or orange flavor. Of course, any material used in preparing any dosage unit 30 form should be pharmaceutically pure and substantially non-toxic in the amounts

employed. In addition, the active compounds may be incorporated into sustained-release preparation and formulations.

Typically, these formulations may contain at least about 0.1% of the active compound or more, although the percentage of the active ingredient(s) may, of course, be varied and may conveniently be between about 1 or 2% and about 60% or 70% or more of the weight or volume of the total formulation. Naturally, the amount of active compound(s) in each therapeutically useful composition may be prepared in such a way that a suitable dosage will be obtained in any given unit dose of the compound. Factors such as solubility, bioavailability, biological half-life, route of administration, product shelf life, as well as other pharmacological considerations will be contemplated by one skilled in the art of preparing such pharmaceutical formulations, and as such, a variety of dosages and treatment regimens may be desirable.

For oral administration the compositions of the present invention may alternatively be incorporated with one or more excipients in the form of a mouthwash, dentifrice, buccal tablet, oral spray, or sublingual orally-administered formulation. For example, a mouthwash may be prepared incorporating the active ingredient in the required amount in an appropriate solvent, such as a sodium borate solution (Dobell's Solution). Alternatively, the active ingredient may be incorporated into an oral solution such as those containing sodium borate, glycerin and potassium bicarbonate, or dispersed in a dentifrice, including: gels, pastes, powders and slurries, or added in a therapeutically effective amount to a paste dentifrice that may include water, binders, abrasives, flavoring agents, foaming agents, and humectants, or alternatively fashioned into a tablet or solution form that may be placed under the tongue or otherwise dissolved in the mouth.

## **II. INJECTABLE COMPOSITIONS AND DELIVERY**

Alternatively, the pharmaceutical compositions disclosed herein may be administered parenterally, intravenously, intramuscularly, or even intraperitoneally as described in U. S. Patent 5,543,158; U. S. Patent 5,641,515 and U. S. Patent 5,399,363

(each specifically incorporated herein by reference in its entirety). Solutions of the active compounds as free base or pharmacologically acceptable salts may be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions may also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils.

- 5 Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions (U. S. Patent 5,466,468, specifically incorporated  
10 herein by reference in its entirety). In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion  
15 medium containing, for example, water, ethanol, polyol (*e.g.*, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and/or vegetable oils. Proper fluidity may be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought  
20 about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

25

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this  
30 connection, sterile aqueous media which can be employed will be known to those of skill

in the art in light of the present disclosure. For example, one dosage may be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

The compositions disclosed herein may be formulated in a neutral or salt form. Pharmaceutically-acceptable salts, include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically

effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug release capsules and the like.

As used herein, "carrier" includes any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

The phrase "pharmaceutically-acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a human. The preparation of an aqueous composition that contains a protein as an active ingredient is well understood in the art. Typically, such compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared. The preparation can also be emulsified.

### **III. Nasal Delivery**

The administration of agonist pharmaceutical compositions by intranasal sprays, inhalation, and/or other aerosol delivery vehicles is also considered. Methods for delivering genes, nucleic acids, and peptide compositions directly to the lungs *via* nasal aerosol sprays has been described *e.g.*, in U. S. Patent 5,756,353 and U. S. Patent 5,804,212 (each specifically incorporated herein by reference in its entirety), and delivery of drugs using intranasal microparticle resins (Takenaga *et al.*, 1998) and lysophosphatidyl-glycerol compounds (U. S. Patent 5,725,871, specifically incorporated herein by reference in its entirety) are also well-known in the pharmaceutical arts. Likewise, transmucosal drug delivery in the form of a polytetrafluoroethylene support

matrix is described in U. S. Patent 5,780,045 (specifically incorporated herein by reference in its entirety).

#### **IV. Additional Modes of Delivery**

5 In addition to the methods of delivery described above, the following techniques are also contemplated as alternative methods of agonist compound delivery. Sonophoresis (*i.e.*, ultrasound) has been used and described in U. S. Patent 5,656,016 (specifically incorporated herein by reference in its entirety) as a device for enhancing the rate and efficacy of drug permeation into and through the circulatory system. Other drug delivery  
10 alternatives contemplated are intraosseous injection (U. S. Patent 5,779,708), microchip devices (U. S. Patent 5,797,898), ophthalmic formulations (Bourlais *et al.*, 1998), transdermal matrices (U. S. Patent 5,770,219 and U. S. Patent 5,783,208) and feedback controlled delivery (U. S. Patent 5,697,899), each specifically incorporated herein by reference in its entirety.

15

#### **G. THERAPY FOR CHOLESTEROL RELATED DISORDERS**

Disorders of lipoprotein metabolism and the susceptibility of human metabolism to adverse effects from diets high in saturated fats have resulted in epidemic atherosclerotic disease in the United States and other developed countries. One aspect of  
20 lipid metabolism, and a primary figure in atherosclerotic disease is cholesterol. Lipoproteins transport cholesterol and triglycerides, which are not water soluble, from sites of absorption and synthesis to sites of utilization. Lipoproteins are classified into six major groups based on size, density, electrophoretic mobility and lipid/protein composition. These six classes are chylomicrons (dietary triglycerides), VLDL  
25 (endogenous triglycerides), IDL (cholesterol ester, triglycerides), LDL (cholesterol ester), HDL (cholesterol ester), and Lp (cholesterol ester) with the major lipid in each group represented parenthetically. There are both exogenous (dietary) and endogenous (primarily liver) sources of cholesterol. Similarly, there is an exogenous lipid transport pathway to transport dietary cholesterol absorbed in the intestine, and an endogenous  
30 pathway to transport cholesterol secreted by the liver.



## I. DISEASED STATES

There are several clinical manifestations associated with lipoprotein disorders due to a breakdown somewhere along the route of lipid metabolism which result in elevated cholesterol levels. The present invention seeks to reduce cholesterol levels by administering therapeutic agents as described herein. Generally, the relevant disease states are those where *in vivo* cholesterol levels are above the desired cut off for the particular clinical situation of the patient. Obviously, the level can vary depending upon the age, gender, genetic background and health of the patient.

Examples of disease suitable for treatment according to the present invention are familial lipoprotein lipase deficiency, an autosomal recessive disorder; familial apolipoprotein C-II deficiency, an autosomal recessive disorder; familial hypertriglyceridemia, an autosomal dominant disorder; familial defective apolipoprotein B-100; and familial combined hyperlipidemia. All of these lipid-related diseases can lead to elevated cholesterol levels. However, the most common cause of high blood cholesterol is due to familial hypercholesterolemia (FH). FH is an autosomal dominant disorder caused by a mutation in the gene encoding the LDL receptor protein. Five classes of mutant alleles have been identified that cause a functional or absolute LDL receptor deficiency. Treatment of all of these diseases is contemplated as part of the present invention. Thus, a step of identifying individuals with these diseases may be included as part of any therapeutic method disclosed herein.

The drugs administered to achieve these therapeutic goals are selected RXR and LXR $\alpha$  agonists that are capable of reducing lipid adsorption and increasing bile acid synthesis, respectively, *in vivo*. Many of these compounds are known for other indications, and may be applied here upon screening for the appropriate activity, as set forth above. For example, the rexinoid LG268 is suitable for treatments aimed at reducing cholesterol adsorption. Additionally compounds that may prove useful may be

found in U. S. Patent 5,455,265 and U. S. Patent 5, 780,676, each specifically incorporated herein by reference in its entirety.

Therapies also may encompass diagnostic procedures to establish the need for a particular therapeutic regimen. Methods for determining cholesterol levels are well known, widely practiced, and many commercial kits are readily available. It also is envisioned that continued monitoring of cholesterol levels throughout a course of treatment will be utilized, both to assess the efficacy of the treatment, and to establish whether an increase or decrease in the drug dosage is required.

## II. COMBINATION THERAPIES

In certain embodiments, the combined administration of cholesterol adsorption inhibitors with other drugs can prove particularly advantageous. Other compounds that may be used in conjunction with RXR agonists that reduce cholesterol adsorption are (a) LXR $\alpha$  agonists, or (b) one or more of the three classes of antilipemic agents currently in use for treating lipid disorders, or both (a) and (b). The three groups of antilipemic drugs are classified as (i) bile acid sequestrants, (ii) fibric acid derivatives and (iii) HMG-CoA reductase inhibitors.

Examples of bile acid sequestrants are Colestid<sup>™</sup>, LoCholest<sup>™</sup>, and Questran<sup>™</sup>. Colestid<sup>™</sup> is colestipol hydrochloride, and is used primarily to reduce LDL-cholesterol. A single dosage is 5 grams of the powdered product, with one or two doses per day at the beginning of treatment, moving to one dose a day. Regimens are one to two months in duration. If necessary, dosage may be increased up to six doses per day in the absence of toxicity. The active ingredient of LoCholest<sup>™</sup> and Questran<sup>™</sup> is cholestyramine resin, and is used as an adjunctive therapy for elevated serum cholesterol. One dose is 9 grams of the powdered product, administered once or twice per day and increasing to 4 doses daily. Increases are recommended at intervals not less than four weeks.

Fibric acid derivatives are Atromid-S™ and Lopid™. Atromid-S™ contains ethyl 2-(p-chlorophenoxy)-2-methyl-propionate and lowers triglyceride levels. Daily dosage for adults is 2 grams, divided into two doses. The active ingredient Lopid™ is gemfibrozil. It acts by reducing serum triglycerides and LDL-cholesterol. Dosage is 600 mg, twice daily, before the morning and evening meals.

HMG-CoA reductase inhibitors are Pravachol™ (pravastatin), Baycol™ (cerivastatin), Lescol™ (fluvastatin), Lipitor™ (atorvastatin) (lovastatin) Mevacor™ (lovastatin), Zocor™ (simvastatin) and other of the so-called “statins.” Dosages for each of these compounds are well known to the skilled artisan and can be found, for example, in the Physician’s Desk Reference, 52nd Ed., MEDICAL ECONOMICS CO. (1998).

These compounds may be given together, or sequentially as part of a treatment regimen. For example, a combination therapy may involve alternating administrations of two agents (A, B, A, B, A, B ....) or three agents (A, B, C, A, B, C, ....). Both of these embodiments are considered variations of combination therapies. Typical intervals between different therapies range from one hour to a week or even a month. However, intervals of about 24 to 48 hours are envisioned as particularly useful.

## H. EXAMPLES

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

## EXAMPLE 1

### Materials and Methods for Preparation and Study of LXR $\alpha$ Knockout Mice

#### Construction of the LXR $\alpha$ Targeting Vector

5        Mouse LXR $\alpha$  genomic clones were obtained by screening a 129Sv  $\lambda$  genomic library using the 1.4 kb full-length cDNA of human LXR $\alpha$  (Willy *et al.*, 1995). The targeting vector was constructed using a neo-TK template plasmid containing a neomycin and two tandem herpes simplex virus thymidine kinase cassettes (Rosahl *et al.*, 1993). This vector was designed to replace exons 3 -6 by homologous recombination, which  
10       contain the complete DNA-binding domain and majority of the ligand-binding domain of LXR $\alpha$  (amino acids 86-327). A short arm was generated by blunt end inserting the 1.9 kb EcoRI-ApaI fragment containing exons 1 and 2 into the unique BamHI site 5' of the neo cassette, and the longer arm by blunt end inserting the 4.0 kb NcoI fragment containing exons 7-9 into the unique XhoI site located between the neo and TK cassettes. The  
15       targeting vector was linearized at a unique Sall site prior to electroporation.

#### Generation of LXR $\alpha$ Null Mice

      Ablation of the mouse *Lxr $\alpha$*  gene locus was accomplished according to previously described methods (Ishibashi *et al.*, 1993; Rosahl *et al.*, 1993). SM-1 mouse ES cells  
20       were grown on irradiated STO feeder cells. Approximately  $5 \times 10^7$  cells were electroporated with 1 mg/ml of linearized targeting vector and subjected to selection with G418 and FIAU. Resistant clones were analyzed by Southern blotting after Hindi digestion. Two positive clones were expanded and injected into C57BL/6 blastocysts to produce five male chimeric mice, which transmitted the mutant gene through the  
25       germline. The mice were genotyped by Southern blotting of tail DNA after EcoRI digestion. Subsequent experimental procedures were performed on the C57BL/6-129Sv mixed strain descendants (F2 and subsequent generations) and repeated with the second clone, with the exception of the 90-day 2% cholesterol diet. The mice were maintained on a 12 hr lighted hr dark cycle and fed ad libitum a standard, cereal-based mouse/rat diet  
30       (No, 7001, Harlan Teklad) containing 4% (w/w) fat and approximately 0.02% (w/w)

cholesterol. Where indicated, this diet was supplemented with 0.2% or 2% (w/w) cholesterol (ICN). The mice were euthanized after a 5 hr fast during the early phase of the light cycle unless otherwise indicated. All mouse procedures used in this study were performed in accordance with the Institutional Animal Care and Research Advisory Committee at the University of Texas Southwestern Medical Center at Dallas.

### Northern Blotting

Total RNA were extracted using RNA STAT-60 (Tel-Test, Inc.), and mRNA isolated using oligo(dT)-cellulose columns (Pharmacia Biotech). Equivalent amounts of mRNA from 4-6 mice were pooled, and 4-10  $\mu$ g subjected to Northern blot analysis as described (Lehrman *et al.*, 1987). Mouse cDNA probes for *Lxr $\alpha$*  and *Lxr $\beta$*  (Lehmann *et al.*, 1997; cholesterol 7 $\alpha$ -hydroxylase (Ishibashi *et al.*, 1996); oxysterol 7 $\alpha$ -hydroxylase (Schwarz *et al.*, 1997); *PPAR $\alpha$* , *PPAR $\beta$* , *PPAR $\gamma$*  (Kliewer *et al.*, 1994); and 15 other lipid regulatory genes (Shimano *et al.*, 1997) were prepared as described. *Sr-BI* cDNA probe was a gift from Dr. Helen Hobbs. Probes for SCAP and sterol carrier protein were prepared by RT-PCR with mouse liver poly(A)<sup>+</sup> RNA as described previously for other probes (Shimano *et al.*, 1996). PCR<sup>TM</sup> primers used were: SCAP, 5'-TTAAGCTTTGTCCCGGGCATTCCAACCTGG-3' (SEQ ID NO:1) and 5'-TTGAATTCGACTTGCTGAGCACCAACACAT-3' (SEQ ID NO:2); and sterol carrier protein, 5'-AAGAAGCTTGAAGAGGAAGGGGAA-3' (SEQ ID NO:3) and 5'-AGCTTAGCTTTGTCCGGCTGAAG -3' (SEQ ID NO:4) (Mori *et al.*, 1991). Northern were quantitated using a phosphorimager (Molecular Dynamics) and standardized against  $\beta$ -actin controls.

### Histological Procedures

Liver, kidney, spleen, adrenal, small intestine, brain, testis, ovary, skin, muscle, white and brown adipose tissue, heart, and lung tissue were fixed in formalin (Accustain; Sigma), embedded in paraffin wax, sectioned, and stained with hematoxylin and eosin by a standard procedure. Frozen tissue sections were stained with oil red O using standard

procedures. Sections were examined under bright-field microscopy with an Olympus model BX50 photomicroscope.

### Plasma and Tissue Chemical Analyses

5 Mice were anaesthetized with sodium pentobarbital and exsanguinated *via* the ascending vena cava. Blood was transferred to tubes containing EDTA (Microcuvette CB1000 capillary tubes; Sarstedt, Inc.), and the plasma isolated by centrifugation. Pooled plasma (from five mice) was analyzed for cholesterol, triglyceride, and lipoproteins as previously described (Ishibashi *et al.*, 1993). Whole livers were removed, weighed, and  
10 sections taken (0.2 g) for lipid analysis. The lipids were extracted and analyzed for triglycerides and cholesterol as previously described (Bucolo and David, 1973; Yokode *et al.*, 1990). Plasma analysis of glucose, aspartate aminotransferase, alanine aminotransferase, cholesterol, triglycerides, albumin, and total protein levels was performed using a Paramax RX automated analyzer (Dade International).

### Bile Acid Analyses

15 In studies involving the measurement of bile acid pool size, composition, and excretion, male mice were housed individually in plastic cages containing wood shavings and were fed their respective diets *ad libitum*. Stools were collected from each animal  
20 over the 72 hr immediately prior to study, dried, weighed, and ground in a mechanical blender. Aliquots were taken for the measurement of total bile acid content by an enzymatic method as described (Turley *et al.*, 1997). The daily stool output (g/day per 100 g body weight) and fecal bile acid content ( $\mu\text{mol/g}$ ) were used to calculate the rate of bile acid excretion (Monday per 100 g body weight). After the mice were weighed,  
25 anesthetized, and exsanguinated, the entire small intestine and its contents, along with the bulk of the liver and the gallbladder, were combined and extracted in ethanol with  $[24\text{-}^{14}\text{C}]$  taurocholic acid (New England Nuclear) added to correct for procedural losses. The total bile acid content of the extract was quantitated by HPLC. These data, together with the respective value for recovery of the internal standard, were used to calculate bile  
30 acid pool size ( $\mu\text{mol}/100\text{ g body weight}$ ) and composition. Irrespective of genotype or

diet, taurocholic acid and tauromuricholic acid accounted for more than 95% of the bile acids in the pool in all cases. No attempt was made to resolve what fraction of the muricholic acid peak corresponded to  $\beta$ ,  $\alpha$ , and  $\omega$  forms of this bile acid.

## 5                    **Cell Culture and Cotransfection Assays**

Transient transfections in Hepa1-6 mouse liver cells were performed as described (Willy *et al.*, 1995). Cells were maintained at 5% CO<sub>2</sub> in DMEM supplemented with 10% fetal bovine serum. Receptor expression plasmids encoding mouse LXR $\alpha$  or LXR $\beta$  in CMX vectors were cotransfected with a luciferase reporter plasmid (TK-CYP7A-LXREx3-LUC) containing three tandem copies of the DR-4 sequence (gcttTGGTCActcaAGTTCAagtta, SEQ ID NO:5) from the rat *Cyp7a* gene (Chiang and Stroup, 1994). 24(S),25-epoxycholesterol was dissolved in ethanol and added to cells after transfection in medium containing 5% cabosil-treated newborn calf serum. All transfections were normalized to a  $\beta$ -galactosidase internal standard as a control. Data are presented as mean relative light units (RLUs) from triplicate assays  $\pm$  SEM.

## **Statistical Analyses**

Values are expressed as mean  $\pm$  standard error of the mean (SEM). The significance of differences between mean values were evaluated using the unpaired Student's *t*-test. Sample groups showing heterogeneity of variance were appropriately transformed before analysis

## **EXAMPLE 2**

### **Targeted Disruption of the *Lxra* Gene**

25

The mouse *Lxra* gene includes nine exons that encompass the translated portion of the gene. Exons 1 and 2 encode the amino-terminal portion of the receptor (A/B regions), exon 3 encodes all of the DNA-binding domain (C region), and exons 4-9 encode the hinge and ligand-binding domains (D and E regions). A targeting construct was prepared by inserting a PGK-neo cassette between an Apal site at the beginning of

5 exon 3 and an NcoI site in the intron between exons 6 and 7. Homologous recombination of this construct in SM-1 ES cells replaced exons 3-6 encoding the DNA-binding and ligand-binding domains of LXR $\alpha$  (amino acids 86-327). Two ES cell clones were chosen for generation of chimeric, heterozygous (LXR $\alpha$  (+/-)) and homozygous (LXR $\alpha$  (-/-)) mice. Mutant mice were viable and fertile, and they externally appeared normal when reared under normal laboratory conditions on standard rodent chow. Under these conditions, heterozygous and homozygous animals lived normal lifespans and bred with predicted Mendelian distributions. Analysis of liver mRNA from these mice revealed that heterozygotes retain significant levels of LXR $\alpha$  transcripts (~50% of wild type), while no wild-type transcripts are detected in homozygous mice. In addition, no LXR $\alpha$  protein was detected in homozygotes by immunoblot analysis using antibodies specific for the hinge region of LXR $\alpha$ . The inventors conclude that this disruption results in mice that are null for LXR $\alpha$ .

### 15 EXAMPLE 3

#### Hepatomegaly and Cholesterol Accumulation in LXR $\alpha$ Null Mice

Previously, the inventors and others have shown that LXRs are activated by a specific group of monooxidized metabolites of cholesterol, including 22(R)-hydroxycholesterol, 24(S)-hydroxycholesterol, and 24,25(S)-epoxycholesterol (Janowski *et al.*, 1996; Lehmann *et al.*, 1997). The fact that these metabolites are naturally occurring and are elevated in tissues where cholesterol metabolism is high (*e.g.*, the liver) led to the prediction that LXRs may have a role in the regulation of cholesterol metabolism. Since wild-type murine species have an extraordinary capacity to ingest large quantities of cholesterol without deleterious effects (Breslow, 1996), one hypothesis the inventors wished to explore was whether LXR $\alpha$  null mice had a defect in their ability to respond to dietary cholesterol. To test that idea, 2-3 month old, gender-matched LXR $\alpha$  wild-type and null mice were characterized after being reared on diets containing different cholesterol concentrations for 0, 7, 30, and 90 days. Analysis of LXR $\alpha$  (-/-) animals fed a standard laboratory rodent chow indicated these mice appeared identical to



their wild-type littermates as determined by morphological and histological studies (see below). In retrospect, the lack of a gross phenotype on this diet in the mutant mice is perhaps not surprising, since standard rodent chow contains relatively little cholesterol ( $\leq 0.02\%$  w/w). On this diet, the cholesterol requirement of these animals is achieved primarily through *de novo* biosynthesis (Osono *et al.*, 1995).

The inventors next treated the effect of a diet rich in cholesterol (chow supplemented with 2% cholesterol). On a per diem basis, the intake of cholesterol on this diet is approximately 20-30 times above the amount an average animal synthesizes to maintain homeostasis when its dietary needs are not being met (Osono *et al.*, 1995). In contrast to wild-type animals, which can sustain this cholesterol-rich diet indefinitely without deleterious effects, there were dramatic morphological, histological, and chemical changes in the livers of LXR $\alpha$  null mice fed the same diet. Within 7 days of beginning the 2% cholesterol diet and chronically worsening over a 90-day period, there was a prominent color and size change in the LXR $\alpha$  (-/-) vs wild-type livers.

Histological examination of these livers demonstrated the presence of a time-dependent increase in the number and size of intracellular vacuoles, characteristic of lipid deposits. Oil red O staining of liver sections verified the deposition of increasing quantities of lipid by 7 and 30 days. After 90 days, the morphology of the LXR $\alpha$  (-/-) livers had been substantially altered, and there was distinct evidence of increased hematopoietic foci, macrophage infiltration, and progressive hepatocellular degeneration. Chemical analysis of these livers showed that, consistent with the distinct color change, all of the accumulated lipid is in the form of cholesterol, 75% of which is esterified. Remarkably, the LXR $\alpha$  null animals exhibited a 15- to 20-fold increase in hepatic cholesterol within just 7 days of feeding the cholesterol-rich diet. Both liver mass and cholesterol concentration continued to increase with time, and by 90 days the LXR $\alpha$  (-/-) livers had doubled in mass and were in the advanced stages of failure. Consistent with the known ability of wild-type animals to adapt quickly to cholesterol-rich diets, the livers of LXR $\alpha$  (+/+) animals maintained essentially normal appearance and function. In these

animals, there were no significant changes in liver histology or size, and only nominal increases in hepatic cholesterol were detected. Interestingly, there was an increase in hepatic triglyceride levels in wild type, but not LXR $\alpha$  null mice. The elevated hepatic triglyceride level seen in the wild-type mice explains the slight pigment change in the  
5 livers of these animals, even though their cholesterol levels remained low.

To confirm the dietary dependence of the defect seen in LXR $\alpha$  (-/-) mice, a second group of wild-type and homozygous animals was analyzed after 7 and 22 days on an intermediate cholesterol diet (chow supplemented with 0.2% cholesterol). On this  
10 diet, the average animal consumes an amount of cholesterol per day that is approximately twice what it needs to maintain normal homeostasis. LXR $\alpha$  (-/-) mice accumulated hepatic cholesterol to levels 3- and 10-fold higher than wild-type mice when fed a 0.2% cholesterol diet for 7 and 22 days, respectively. A comparison of mice on the 0.2% cholesterol diet vs. mice on the 2% cholesterol shows that the defect in LXR $\alpha$  (-/-) mice  
15 causes a near linear accumulation of hepatic cholesterol, regardless of whether the diet contains 0.2% or 2% cholesterol. These results demonstrate that, in the absence of LXR $\alpha$ , mice are unable to tolerate any dietary cholesterol in excess of what they can synthesize *de novo*.

Similar dietary studies were performed using heterozygous mice, which express approximately half of the levels of LXR $\alpha$ . These mice displayed an intermediate phenotype, accumulating hepatic cholesterol concentrations to levels between those observed for LXR $\alpha$  (+/+) and (-/-) mice. These results indicate that expression levels of LXR $\alpha$  are crucial for a complete response.  
20

Significantly, there were no changes in the body weights of either LXR $\alpha$  (-/-) or (+/+) mice, irrespective of the type or length of the diet. However, there was a noticeable decrease in the amount of white adipose tissue in LXR $\alpha$  (-/-) mice after 90 days on the 2% cholesterol diet, which likely compensated for the net increase in liver mass.  
25

Morphological and histological examinations of several other tissues where LXR $\alpha$  is  
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expressed, including kidney, small intestine, spleen, and adrenal, are ongoing, but preliminary results have revealed no obvious abnormalities in these tissues thus. To date, no gender-specific phenotypic differences have been observed in LXR $\alpha$  knockout mice, although female mice tend to have higher basal and diet-induced cholesterol levels.

5

Analysis of the plasma from the animals used in the above dietary studies confirms and extends the conclusion that LXR $\alpha$  null animals have a crucial defect in hepatic metabolism of dietary cholesterol (Table 3). Plasma cholesterol levels rose significantly (>200%) over the course of the 90-day, high-cholesterol diet in LXR $\alpha$  (-/-) mice, while wild-type mice characteristically showed no change. There was also a dramatic increase in the levels of alanine and aspartate amino transferases (26- and 7-fold, respectively, within 30 days) in the serum of the LXR $\alpha$  (-/-), but not wild-type animals. These enzymes are predominantly expressed in the liver, and the large increase in their levels in serum is indicative of hepatocyte injury and necrosis and is consistent with the results of the histological studies. By 90 days, the LXR $\alpha$  (-/-) livers deteriorated to the point where they have lost much of their normal cell structure, and this change was reflected by the observed decrease (between 30 and 90 days) in the levels of amino transferases still present in the serum of these animals (Table 3). Commensurate with impaired liver function, LXR $\alpha$  null mice exhibited a time-dependent decrease in serum glucose to half the normal level of their wild-type littermates when fed the high-cholesterol diet. Although several other parameters in the plasma, including triglycerides, total protein, albumin, creatinine, and electrolytes appeared normal, these results demonstrate that significant liver damage has occurred by 90 days and suggest that extension of this diet will ultimately result in complete liver failure.

25

A characteristic of murine species is their ability to regulate intermediate and low-density lipoprotein (IDL/LDL) cholesterol in serum tightly even under prolonged conditions of elevated cholesterol intake. Since the data from Table 3 revealed an increase in plasma cholesterol in the LXR $\alpha$  null mice, a plasma lipoprotein profile was generated from wild-type and homozygous mice fed standard or 2% cholesterol chow

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diets for 30 days. As predicted, wild-type animals were resistant to large increases in dietary cholesterol and maintained very low circulating levels of IDL/LDL cholesterol. However, LXR $\alpha$  (-/-) animals produced an ~5-fold increase in IDL/LDL cholesterol and only minimal changes in HDL when compared to LXR $\alpha$  (+/+) animals fed the 2% cholesterol-supplemented diet.

**TABLE 3**  
**Plasma Analysis of Wild Type and LXR $\alpha$  (-/-) after 2% Cholesterol Diets**

Test	Genotype	0 Days	7 Days	30 days	90 Days
Cholesterol	+/+	84	82	105	82
(mg/dL, $\pm$ <3.5%)	-/-	72	127	162	145
Alanine transaminase	+/+	17	19	13	15
(U/L, $\pm$ <5.5%)	-/-	12	98	314	188
Aspartate transaminase	+/+	39	44	41	45
(U/L, $\pm$ 7%)	-/-	44	160	303	197
Glucose	+/+	231	247	200	219
(m/dL, $\pm$ <3%)	-/-	178	195	109	121
Triglycerides	+/+	52	38	39	43
(mg/dL, $\pm$ <5%)	-/-	46	35	29	31
Total protein	+/+	4.7	ND	4.4	4.4
(g/dL, $\pm$ <4%)	-/-	4.6	4.7	5.4	4.7
Albumin	+/+	1.7	1.5	1.5	1.5
(g/dL, $\pm$ <3%)	-/-	1.6	1.6	1.6	1.4

10

#### EXAMPLE 4

##### Cholesterol and Bile Acid Metabolism Defects in LXR $\alpha$ Null Mice

5 The phenotype described above provides convincing evidence that one of the roles of LXR $\alpha$  is to regulate dietary cholesterol metabolism in the liver. Since the primary pathway for catabolic elimination of cholesterol is through bile acid metabolism, the inventors next examined the response of several important components in this pathway. An 8-day dietary study similar to the one described above was performed on 3-month-old male mice, comparing wild-type and LXR $\alpha$  (-/-) animals fed standard or 2% cholesterol-supplemented chow. The animals in this group were analyzed for rates of fecal bile acid excretion, and bile acid pool size and composition. Similar to the results obtained above, LXR $\alpha$  (-/-) animals manifest a 16-fold increase in hepatic cholesterol when fed a 2% cholesterol chow diet.

15 Within 8 days, the concentration of hepatic cholesterol rose in LXR $\alpha$  null mice from its normal value of  $2.46 \pm 0.05$  mg/g to  $40.09 \pm 0.82$  mg/g tissue, whereas hepatic cholesterol in wild-type mice increased nominally from  $2.21 \pm 0.03$  mg/g to  $5.03 \pm 0.19$  mg/g tissue. Interestingly, even on the low-cholesterol diet there was a statistically significant difference ( $P < 0.001$ ) between the hepatic cholesterol concentration in LXR $\alpha$  (+/+) and (-/-) mice ( $2.21 \pm 0.03$  and  $2.46 \pm 0.05$  mg/g, respectively). These phenotypes were independent of gender.

25 The ability of wild-type animals to maintain a relatively low hepatic cholesterol concentration when cholesterol intake is high is largely due to the compensatory increase in bile acid synthesis. The 123% increase in fecal bile acid excretion and 36% increase in bile acid pool size were substantial and expected, based on previous studies showing that bile acid metabolism is responsible for metabolic clearance of dietary cholesterol (Carey and Duane, 1994; Schwarz *et al.* In marked contrast, the bile acid pool size of the LXR $\alpha$  (-/-) mice was significantly lower than wild-type mice and did not change when the animals were fed the cholesterol-rich diet. In addition, bile acid excretion in the LXR $\alpha$

(-/-) mice increased less than half the amount it did in wild-type animals. Since ordinarily bile acid excretion reflects bile acid synthesis, these results imply that LXR $\alpha$  null mice have a decreased ability to up-regulate bile acid synthesis.

5           An analysis of bile acid pool composition revealed a striking difference in this parameter as well. Over 95% of the murine biliary bile acid pool is composed of cholic and muricholic acids (Akiyoshi *et al.*, 1986). The ratio of cholic acid to muricholic acid is tightly regulated and has a significant effect on the animal's ability to absorb cholesterol in the intestine; the higher the ratio, the more cholesterol that is potentially capable of  
10       being absorbed (Cohen *et al.*, 1977; Uchida *et al.*, 1980). As shown in, wild-type animals respond to increased dietary cholesterol by substantially lowering this ratio (from  $1.34 \pm 0.08$  to  $0.88 \pm 0.05$ ). In contrast, LXR $\alpha$  (-/-) mice have a significantly higher basal ratio ( $2.03 \pm 0.23$ ), which is completely unresponsive to increased cholesterol ( $2.21 \pm 0.57$ ). The above results are consistent with the notion that LXR $\alpha$  (-/-) mice lack a  
15       cholesterol-sensing mechanism that governs the clearance of excess cholesterol through bile acid synthesis.

#### EXAMPLE 5

##### Regulation of *Cyp7a* by LXR $\alpha$

20           The rate-limiting step in the classical bile acid synthesis pathway utilizes the liver-specific enzyme cholesterol 7 $\alpha$ -hydroxylase (Cyp7a), which converts cholesterol into 7 $\alpha$ -hydroxycholesterol (Russell and Setchell, 1992). In marine species, this enzyme is transcriptionally regulated by dietary cholesterol (Rudling, 1992), and the inventors and  
25       others have shown that the *Cyp7a* promoter contains a functional LXR response element (*i.e.*, LXRE) (Lehmann *et al.*, 1997; see also below). In addition to Cyp7a, a second enzyme called oxysterol 7 $\alpha$ -hydroxylase (Cyp7b) exists as part of an alternative bile acid synthesis pathway, which is not transcriptionally regulated by cholesterol (Schwarz *et al.*, 1997). Therefore, it was of interest to characterize the response of these genes in the  
30       LXR $\alpha$  (-/-) mice. As expected, Cyp7a mRNA was up-regulated (5.9-fold) in wild-type

mice fed a high-cholesterol diet for 7 days. However, there was no cholesterol-induced up-regulation of Cyp7a in LXR $\alpha$  (-/-) mice, consistent with the notion that this gene is a direct target of LXR $\alpha$  action. In contrast to Cyp7a, there were no differences in expression of Cyp7b in either LXR (+/+) or (-/-) mice.

5

In addition to normally expressing LXR $\alpha$ , the liver also expresses the closely related receptor, LXR $\beta$ . Since both of these receptors have similar ligand specificities (Janowski *et al.*, 1996; Lehmann *et al.*, 1997), the inventors wished to address why LXR $\beta$  cannot rescue the bile acid phenotype in LXR $\alpha$  (-/-) mice. The expression of the *Lxr $\alpha$*  and  $\beta$  genes is not regulated by dietary cholesterol, indicating that the LXRs are not likely to be auto-regulated in the liver. More importantly, there was no compensatory increase in the level of hepatic LXR $\beta$  in LXR $\alpha$  (-/-) mice. Furthermore, cotransfection studies with LXR $\alpha$  and  $\beta$  expression plasmids demonstrate that the LXRE found in the *Cyp7a* promoter is a substantially stronger response element for LXR $\alpha$  than it is for LXR $\beta$ . These results corroborate the previous findings of Lehmann and coworkers, showing that LXR $\alpha$  has a substantially higher affinity than LXR $\beta$  for binding to the *Cyp7a* LXRE (Lehmann *et al.*, 1997). Taken together with the striking phenotype of LXR $\alpha$  (-/-) mice, the inventors conclude that LXR $\alpha$  and  $\beta$  can differentially regulate gene expression and, thus, may have non-overlapping roles.

20

## EXAMPLE 6

### Impaired Expression of Hepatic Genes Involved in Multiple Lipid Pathways

In addition to the genes involved in bile acid metabolism, the expression of 18 other genes involved in cholesterol and fatty acid metabolism were investigated by Northern blot analysis. End product repression of cholesterol biosynthesis is a well-studied pathway that is controlled primarily by the transcription factor SREBP-2 (Brown and Goldstein, 1997). As expected, the liver mRNA levels of SREBP-2, as well as the cholesterologenic enzymes, hydroxymethyl glutaryl-coenzymeA (HMG-CoA) synthase and reductase, farnesyl diphosphate (FPP) synthase, and squalene synthase were

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down-regulated in response to cholesterol in both LXR $\alpha$  (+/+) and (-/-) animals. Surprisingly, however, there was a significant increase in the expression of these genes in the LXR $\alpha$  (-/-), but not wild-type, animals fed a low cholesterol diet. At present, the consequence of the altered up-regulation of these genes is not fully understood and will require further study; however, the lack of a severe phenotype under low dietary cholesterol conditions suggests this misregulation may not be deleterious. Furthermore, no differences were seen in the regulation of SREBP cleavage activating protein (SCAP), sterol carrier protein, LDL receptor, HDL receptor (SR-B1), or apolipoprotein gene expression in the LXR $\alpha$  null mice.

In contrast to the up-regulation of genes involved in cholesterol biosynthesis, three genes involved in fatty acid metabolism, SREBP-1, stearoyl CoA desaturase-1, and fatty acid synthase, were markedly down-regulated in LXR $\alpha$  (-/-) mice on the low-cholesterol diet. Again, the consequence of the altered expression of these genes on this diet does not appear to be deleterious, but further study will be required to determine if there are other metabolic defects in these mice under other dietary conditions (*e.g.*, low or high fatty acid supplemented diets). The expression of several other genes, including the three peroxisome proliferator activated receptors (PPARs) and acetyl CoA carboxylase were unchanged in the mutant vs wild-type mice.

#### EXAMPLE 7

##### **LG268 prevents liver accumulation of cholesterol in both LXR $\alpha$ wild-type and knockout mice strains.**

Male, A129 strain mice (LXR $\alpha$  +/+ and -/-) were fed Teklad 7001 powdered diet supplemented with 0.2% cholesterol .015% LG268 (provides 30 mg/kg body weight), vehicle, or 2% surfomer (a surfactant polymer that inhibits cholesterol absorption, Turley *et al.*, 1991) ad libitum for 10 days. Animals were sacrificed and a liver aliquot was saponified and extracted and the cholesterol content was determined by gas chromatography using stigmasterol as an internal standard (Turley *et al.*, 1994). Liver



cholesterol accumulation was prevented by ingestion of LG268 in both LXR $\alpha$  knockout and wild-type mice. The two most likely mechanisms for this effect are (1) increased cholesterol output by enhanced bile acid production and secretion and/or (2) decreased cholesterol input by diminished cholesterol absorption.

5

#### EXAMPLE 8

##### **LG268 does not prevent liver accumulation of cholesterol by increasing bile acid production and excretion.**

10 Female, mixed strain (LXR $\alpha$  +/+ and -/-) mice were fed Teklad 7001 2 % cholesterol (w/w) diet ad libitum for 7 days. Each day mice received a single bolus dose of LG268 (200 l/25 g mouse to provide 30 mg/kg body weight) or vehicle by oral gavage. Mice were killed and liver cholesterol was determined as described in figure 1. Small intestine (with luminal contents) and gall bladder were extracted together to determine  
15 bile acid pool size and composition as described by Schwartz *et al.*, 1998. Stools were collected over 3 days from the individually housed mice, dried, weighed and ground. Fecal bile acid content was determined by an enzymatic method (Turley *et al.*, 1996). LG268 did not prevent the liver accumulation by increasing bile acid production (as reflected in bile acid pool size and excretion).

20

#### EXAMPLE 9

##### **LG268 inhibits cholesterol absorption in LXR $\alpha$ wild-type and knockout mouse strains.**

25 Male, A129 strain mice (LXR $\alpha$  +/+ and -/-) were fed Teklad 7001 powdered diet supplemented with 0.2% cholesterol .015% LG268 (provides 30 mg/kg body weight), vehicle, or 2% surfomer ad libitum for 10 days. On day 7, mice received a gavage dose of [22,23-3H]b-sitostanol (American Radiolabeled Chemicals, St. Louis, MO) and [4-14C]cholesterol (Amersham) for the measurement of cholesterol absorption by the fecal

isotope ratio method (Turley *et al.*, 1994). Cholesterol absorption was completely inhibited in mice receiving a dose of 30 mg/kg body weight over 10 days, regardless of LXR $\alpha$  genotype. LG268 was more effective than surfomer, at a dose 130-fold lower than the surfomer administration. In light of the extreme potency of LG268, a dose-response analysis was performed.

#### EXAMPLE 10

**LG268 inhibits cholesterol absorption in wild-type mice at levels as low as 1.4 mg/kg body weight (equivalent to .0007% of the diet (w/w)).**

Male, A129 strain mice were fed Teklad 7001 powdered diet supplemented with 0.2% cholesterol and various levels of LG268 (providing 1.4 to 30 mg/kg body weight) or vehicle ad libitum for 10 days. On day 7, mice received a gavage dose of [22,23-<sup>3</sup>H]b-sitostanol (American Radiolabeled Chemicals, St. Louis, MO) and [4-<sup>14</sup>C]cholesterol (Amersham) for the measurement of cholesterol absorption by the fecal isotope ratio method (Turley *et al.*, 1994). Liver cholesterol concentration was determined as by gas chromatography. LG268 is effective at doses as low as 1.4 mg/kg body weight (.0007 % of the diet) in preventing liver accumulation of cholesterol by inhibiting cholesterol absorption.

#### EXAMPLE 11

**LG268 inhibits cholesterol absorption in wild-type mice while exerting little or no effect on total lipid absorption.**

Total lipid absorption was determined in the study described above (figure 4) to establish whether LG268 effects were specific to cholesterol or were affecting lipid absorption or intestinal integrity. Total lipid absorption was determined gravimetrically (Schwartz *et al.*, 1998) by establishing lipid intake (amount of food consumed over 72 hours x dietary lipid content) and lipid output (fecal lipid content x quantity of stool collected over the same 72 hours) to calculate the fraction of lipid consumed that was

absorbed. Initial observations suggested that mice receiving LG268 were not experiencing general lipid malabsorption or steatorrhea. Stool output was normal, and the feces did not appear greasy or malodorous. The gravimetric determination of lipid absorption shown in figure 5 confirmed these findings. The reduction in lipid absorption seen in these animals can be accounted for by the reduction in cholesterol uptake (cholesterol comprises ~5% of the lipid in these diets and is extracted and measured as lipid in these analyses). Therefore, the effects of LG268 are specific for cholesterol absorption.

## EXAMPLE 12

### **LG268 is exerting its effects on cholesterol absorption through an RXR-mediated pathway.**

Several agents that inhibit cholesterol absorption have been found to affect the physicochemical nature of the intestinal lumen, for example saponins precipitate cholesterol thereby making it unavailable for absorption. It would be unlikely that at the extremely low doses at which LG268 exerts its effects it could be causing such physicochemical changes. However, to rule out this possibility, a cholesterol absorption study was performed using LG268 (which binds and activates RXR) and LG721 (a structurally related compound that binds but fails to activate RXR). If LG268 was causing cholesterol precipitation or micellar disruption in the intestinal lumen, the structural analog LG721 would be predicted to do the same.

Male, A129 strain mice were fed Teklad 7001 powdered diet supplemented with 0.2% cholesterol LG268 or LG721 (to provide 3 mg/kg body weight) ad libitum for 10 days. On day 7, mice received a gavage dose of [22,23-3H]b-sitostanol (American Radiolabeled Chemicals, St. Louis, MO) and [4-14C]cholesterol (Amersham) for the measurement of cholesterol absorption by the fecal isotope ratio method (Turley *et al.*, 1994). Liver cholesterol concentration was determined as by gas chromatography. LG721 fails to prevent liver cholesterol accumulation or inhibit cholesterol absorption. This

would suggest that the activity of LG268 relies not on the small lipophilic nature of the molecule (shared by LG721), but by its unique capacity to bind and activate the retinoid X receptor.

5

### EXAMPLE 13

**LG268 inhibits cholesterol absorption so effectively that mice compensate for the lack of exogenous cholesterol by raising production of endogenous cholesterol in liver and other organs.**

10 Male, A129 mice were fed Teklad 7001 diet supplemented with 0.2% cholesterol and vehicle or LG268 (at 3 or 30 mg/kg body weight) for 7 days ad libitum. Rates of sterol synthesis were measured in vivo as described (Jeske and Dietschy, 1980, Turley *et al.*, 1981) based on the incorporation of [<sup>3</sup>H]water into digitonin-precipitable sterols over one hour. Mice treated for 7 days with LG268 exhibit increased cholesterol biosynthesis to compensate for the absense of an exogenous supply due to a block in cholesterol  
15 absorption. Increased synthesis was also observed in spleen, kidney, and carcass and in all tissues was LG268 dose-dependent and inversely correlated to cholesterol absorption capacity.

20

### EXAMPLE 14

**LG268 prevents liver cholesterol accumulation and inhibits cholesterol absorption in the Golden Syrian hamster.**

25 Hamsters provide a better animal model for cholesterol metabolism than mice (however, mice offer the advantage that they can be genetically manipulated, for example the LXR $\alpha$  -/- strain). Like humans, hamsters carry the bulk of their blood-borne cholesterol in LDL particles, and do not greatly respond to increased dietary cholesterol by increasing bile acid production and excretion. Male Syrian Golden hamsters were fed Teklad 7001 diet supplemented with 0.2% cholesterol and vehicle, LG268 (providing 3 or  
30 30 mg/kg body weight) or 2% surfomer. On day 7, they received a gavage dose of

[22,23-3H]b-sitostanol (American Radiolabeled Chemicals, St. Louis, MO) and [4-14C]cholesterol (Amersham) for the measurement of cholesterol absorption by the fecal isotope ratio method (Turley *et al.*, 1994). Stools were collected from the individually housed hamsters over the next 72 hours. On day 12 the animals were sacrificed and liver was removed for cholesterol determination by gas chromatography (Turley *et al.*, 1994). LG268 was effective in hamsters in reducing liver cholesterol and inhibiting cholesterol absorption. Therefore the effects of LG268 on cholesterol homeostasis are not limited to mice, but observed in other mammalian species as well.

## EXAMPLE 15

### **LG268 does not regulate the expression of several genes proposed to affect cholesterol uptake and processing by the intestine.**

It is known that exogenous cholesterol is taken up in the distal duodenum (and perhaps proximal jejunum) by the intestinal enterocyte, is rapidly esterified (by ACAT) and packaged into chylomicrons for transport into the lymphatic system (reviewed by Homan and Krause, 1997). Recently, caveolin has also been proposed to play a role in cholesterol trafficking in the intestine (Field *et al.*, 1998). Disruption of any of these processes could result in diminished cholesterol absorption capacity. As LG268 is an agonist of the nuclear hormone receptor RXR, it is likely that the primary targets of this drug would be transcriptionally regulated and show a change in the steady state level of mRNA for critical target genes. Northern analyses were performed on duodenal intestine samples obtained in the experiment described in figure 4. Results were quantitated using a phosphoimager (Molecular Dynamics), standardized against b-actin controls, and mathematically adjusted to yield 1 unit for the vehicle-treated control group. No apparent change in ACAT-1, ACAT-2 or caveolin mRNA levels was observed in LG268-treated mice. This suggests that LG268 acts upon other novel gene targets involved in intestinal cholesterol metabolism. Since the mechanism of cholesterol absorption is unknown and is one of the key pathways regulating cholesterol homeostasis, these findings suggest

rexinoids will be powerful tools to dissect the regulatory events that govern this important metabolic pathway.

#### EXAMPLE 16

##### 5      **LG268 regulates the expression of the ATP-binding cassette protein-1 (ABC-1).**

10      The ATP-binding cassette protein-1 is a newly described membrane protein implicated in reverse cholesterol transport, in other words the efflux of cholesterol from peripheral tissues to the liver (Young and Fielding, 1999; Tall, 1998). Defects in the *ABCI* gene are responsible for Tangiers disease, a rare genetic disorder in which patients exhibit cholesterol-laden macrophages, premature atherosclerosis, and the complete absence of circulating HDL (Brooks-Wilson *et al.*, 1999; Bodzioch *et al.*, 1999; Rust *et al.*, 1999). While it has been firmly established that ABC-1 is expressed in macrophage cells, the inventors now demonstrate that ABC-1 is produced in the mouse small intestine in a cephalocaudal distribution that corresponds to regions known to be involved in cholesterol absorption (FIG. 10A). Most importantly, ABC-1 expression is greatly increased in mice orally dosed with LG268 (30 mg/kg body weight) for as little as 6 hours (FIG. 10A and FIG. 10B).

20      Male A129 strain mice were fed Teklad 7001 powdered diet supplemented with 0.2% cholesterol and .015% LG268, equimolar LG721 (see example 12) or vehicle at 7 pm (beginning of dark cycle). Animals were sacrificed at 1 am (6 hour time point) or 7 am (12 hr), the intestine was removed and rinsed with ice-cold phosphate buffered saline, then subdivided into 5 segments of equal length (~8 cm each, Figure 10A) or 3 segments (the proximal segment representing the duodenumis depicted in FIG. 10B). Intestinal mucosa was obtained from each segment and poly (A+) RNA isolated for northern analyses. Results were quantified using a phosphorimager, standardized against b-actin controls, and mathematically adjusted to yield 1 unit for the vehicle-treated control group.



## REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

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**WHAT IS CLAIMED IS:**

- 5
1. A non-human transgenic mammal, the cells of which comprise at least one non-functional endogenous LXR $\alpha$  allele.
2. The non-human transgenic mammal of claim 1, wherein said cells comprise two non-functional endogenous LXR $\alpha$  alleles.
- 10
3. The non-human transgenic mammal of claim 1, wherein said mammal is selected from the group consisting of mouse, rat, hamster, guinea pig, rabbit, cow, and sheep.
- 15
4. The non-human transgenic mammal of claim 1, wherein said non-functional LXR $\alpha$  allele contains an interruption in the LXR $\alpha$  coding sequence.
5. The non-human transgenic mammal of claim 2, wherein said non-functional LXR $\alpha$  alleles both contain an interruption in the LXR $\alpha$  coding sequences.
- 20
6. The non-human transgenic mammal of claim 1, wherein said non-functional LXR $\alpha$  allele contains a nonsense mutation that truncates the LXR $\alpha$  product.
7. The non-human transgenic mammal of claim 2, wherein said non-functional LXR $\alpha$  alleles both contain a nonsense mutation that truncates the LXR $\alpha$  products.
- 25
8. The non-human transgenic mammal of claim 1, wherein said non-functional LXR $\alpha$  allele contains a deletion of LXR $\alpha$  coding sequences.
9. The non-human transgenic mammal of claim 2, wherein said non-functional LXR $\alpha$  alleles both contain a deletion of LXR $\alpha$  coding sequences.
- 30



10. The non-human transgenic mammal of claim 1, wherein said non-functional allele contains an alteration in the regulatory region of the LXR $\alpha$  gene.
11. The non-human transgenic mammal of claim 2, wherein said non-functional LXR $\alpha$  alleles both contain an alteration in the regulatory region of the LXR $\alpha$ s.
12. The non-human transgenic mammal of claim 10, wherein said alteration comprises substitution of an inducible/repressable promoter for the endogenous LXR $\alpha$  promoter.
13. The non-human transgenic mammal of claim 11, wherein said alterations comprise substitution of inducible/repressable promoters for both of the endogenous LXR $\alpha$  promoters.
14. The non-human transgenic mammal of claim 1, wherein cells of said mammal further comprise an exogenous selectable marker gene under the control of a promoter active in at least one cell type of said mammal.
15. A method for screening an RXR agonist or LXR $\alpha$  agonist candidate substance for the ability to increase bile acid synthesis comprising:
  - (a) providing a cell;
  - (b) contacting said cell with said candidate substance; and
  - (c) monitoring a bile acid-related phenotype of said cell,wherein an increase in said bile acid-related phenotype in said cell treated with said candidate substance, as compared to a similar cell not treated with said candidate substance, indicates that said candidate substance increases bile acid synthesis.

16. The method of claim 15, wherein said cell is a liver cell.
17. The method of claim 15, wherein said bile acid-related phenotype is expression of a gene involved in bile acid synthesis.
- 5 18. The method of claim 17, wherein said gene is *Cyp7a*.
19. The method of claim 15, wherein said candidate substance is an RXR agonist.
- 10 20. The method of claim 15, wherein said RXR agonist is a rexinoid.
21. A method for screening a candidate substance for the ability to reduce cholesterol levels in a mammal comprising:
- 15 (a) providing a non-human transgenic mammal, the cells of which comprise at least one non-functional endogenous LXR $\alpha$  allele;
- (b) treating said mammal with said candidate substance; and
- (c) monitoring a cholesterol-related phenotype in said mammal,
- 20 wherein a reduction in said cholesterol-related phenotype in mammals treated with said candidate substance, as compared to a similar mammal not treated with said candidate substance, indicates that said candidate substance reduces cholesterol levels.
- 25 22. The method of claim 21, wherein said mammal is selected from the group consisting of mouse, rat, hamster, guinea pig, rabbit, cow, and sheep.
23. The method of claim 21, wherein said phenotype is cholesterol absorption, circulating cholesterol, hepatic cholesterol, hepatomegaly, atherosclerosis, cardiac

failure, cardiac (atrophy/hypertrophy), activity level, survival, cancer, reproduction, immune function, skin disease, cognitive function, and adrenal function.

5 24. The method of claim 21, wherein said mammal is maintained on a high cholesterol diet.

25. The method of claim 21, wherein said mammal further is treated with an agent that blocks cholesterol biosynthesis.

10 26. The method of claim 21, wherein said cells comprise two non-functional endogenous LXR $\alpha$  alleles.

15 27. A method for screening a candidate substance for the ability to increase bile acid synthesis in a mammal comprising:

- (a) providing a non-human transgenic mammal, the cells of which comprise at least one non-functional endogenous LXR $\alpha$  allele;
- (b) treating said mammal with said candidate substance; and
- (c) monitoring a bile acid-related phenotype in said mammal

20 wherein an increase in said bile acid-related phenotype in mammals treated with said candidate substance, as compared to a similar mammal not treated with said candidate substance, indicates that said candidate substance increases bile acid synthesis.

25 28. The method of claim 27, wherein said mammal is selected from the group consisting of mouse, rat, hamster, guinea pig, rabbit, cow, and sheep.

29. The method of claim 27, wherein said bile acid-related phenotype is selected from the group consisting of cholesterol level, Cyp7a synthesis, fecal bile acid excretion, bile acid pool size and bile acid composition.
- 5 30. A method for screening a rexinoid for the ability to inhibit cholesterol absorption by an intestinal cell comprising:
  - (a) providing an intestinal cell;
  - (b) treating said cell with said rexinoid; and
  - 10 (c) monitoring cholesterol absorption by said cell,wherein a reduction in cholesterol absorption by said cell treated with said rexinoid, as compared to a similar cell not treated with said rexinoid, indicates that said rexinoid is an inhibitor of cholesterol absorption.
- 15 31. The method of claim 30, wherein said cell is an duodenal cell.
32. The method of claim 30, wherein said cell is located in a mammal.
- 20 33. The method of claim 30, further comprising comparing the effect of said candidate substance on cholesterol absorption on a cell comprising one or two non-functional endogenous LXR $\alpha$  alleles.
34. A method of reducing cholesterol levels in a mammal comprising the step of
- 25 treating said mammal with an RXR agonist.
35. The method of claim 34, wherein said agonist is a rexinoid.
36. The method of claim 34, further comprising treating said mammal with an agent
- 30 that inhibits cholesterol biosynthesis.

37. The method of claim 35, wherein said agent is HMG CoA reductase inhibitor.
38. The method of claim 34, wherein said mammal is a human.
39. The method of claim 34, further comprising stimulating bile acid synthesis in said mammal.
40. The method of claim 34, further comprising reducing cholesterol intake by said mammal.
41. A method for inhibiting cholesterol absorption in a mammal comprising treating said mammal with said with an RXR agonist.
42. The method of claim 41, wherein said agonist is a rexinoid.
43. The method of claim 41, wherein said mammal is a human.
44. A transgenic cell which comprises at least one non-functional endogenous  $LXR\alpha$  allele.
45. The transgenic cell of claim 44, wherein said cell comprises two non-functional endogenous  $LXR\alpha$  alleles.
46. A rexinoid compound that inhibits cholesterol absorption, identified by a process comprising:
  - (a) providing an intestinal cell;
  - (b) treating said cell with said rexinoid; and
  - (c) monitoring cholesterol absorption by said cell,

wherein a reduction in cholesterol absorption by said cell treated with said rexinoid, as compared to a similar cell not treated with said rexinoid, identified said rexinoid as an inhibitor of cholesterol absorption.

5

47. A rexinoid compound that inhibits cholesterol absorption, produced by a process comprising:

10

- (a) providing an intestinal cell;
- (b) treating said cell with said rexinoid;
- (c) monitoring cholesterol absorption by said cell, wherein a reduction in cholesterol absorption by said cell treated with said rexinoid, as compared to a similar cell not treated with said rexinoid, identified said rexinoid as an inhibitor of cholesterol absorption; and
- (d) producing said rexinoid compound.

15

48. A method of screening for a modulator of ABC1 expression comprising:

20

- (a) providing a cell expressing an RXR;
- (b) contacting said cell with a rexinoid and a candidate substance; and
- (c) determining the expression of ABC1 in said cell,

wherein a change in expression of ABC1, as compared to a cell of step (b), indicates that said candidate substance is a modulator of ABC1 expression.

25

49. The method of claim 48, wherein ABC1 expression is measured by RNA analysis.

50. The method of claim 49, wherein said RNA analysis is Northern analysis or PCR.

- 25

## ABSTRACT

5 The present invention relates to compositions and methods for reducing  
cholesterolemia and its effects. More specifically, the invention is directed, in one  
embodiment, to methods for screening for compounds that affect cholesterol levels  
generally, and in particular, that affect the absorption of cholesterol. The invention also is  
directed to methods of screening for compounds that increase bile acid synthesis. In so  
doing, the inventors describe useful transgenic cells and animals which lack one or both  
alleles of the LXR $\alpha$  gene. Also provided are therapeutic methods designed to reduce  
10 cholesterol levels in suitable subjects. The reduction may be effected by decreasing  
cholesterol absorption, increasing bile acid synthesis, or combinations thereof.  
Particularly useful in decreasing cholesterol absorption are RXR agonists, for example,  
rexinoid compounds. Therapeutic intervention in cholesterol biosynthesis and diet are  
additional adjunct therapies. In addition, the present invention relates to candidate  
15 compounds that modulate the expression of ABC-1 in a cell that expresses RXR.  
Methods of identifying and making a modulator of ABC-1 are disclosed.



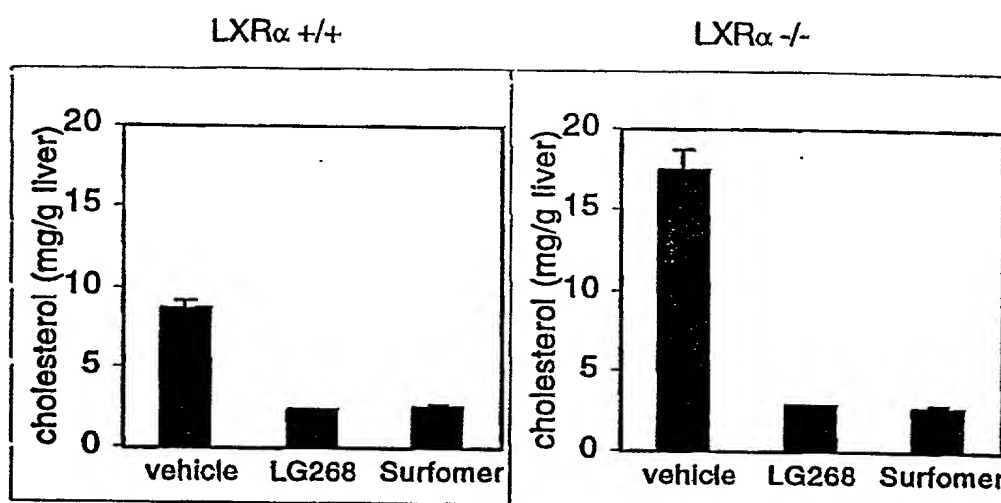


FIG. 1

# Liver cholesterol

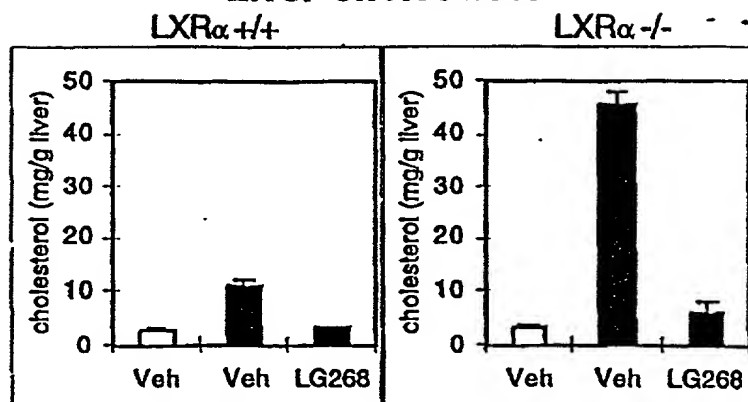


FIG. 2A

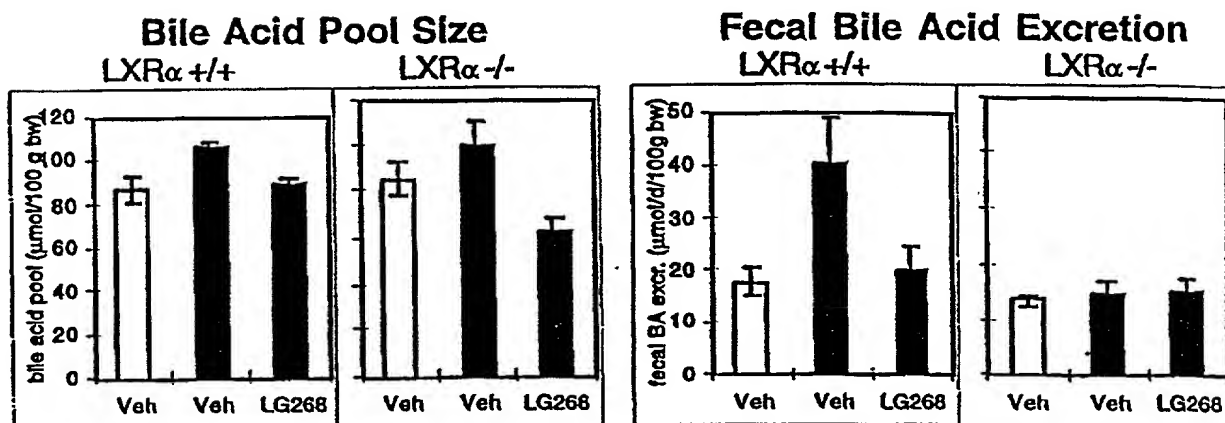


FIG. 2B

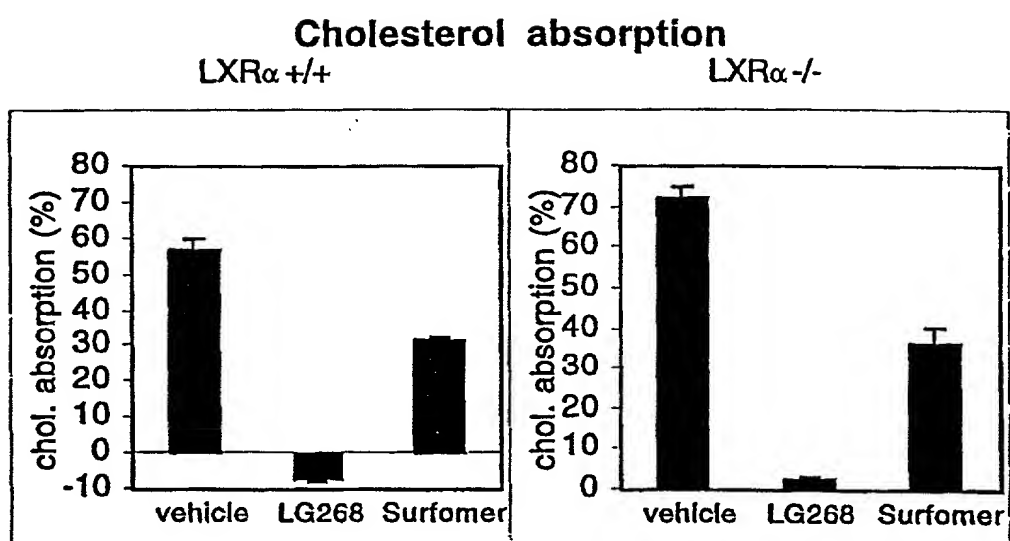


FIG. 3

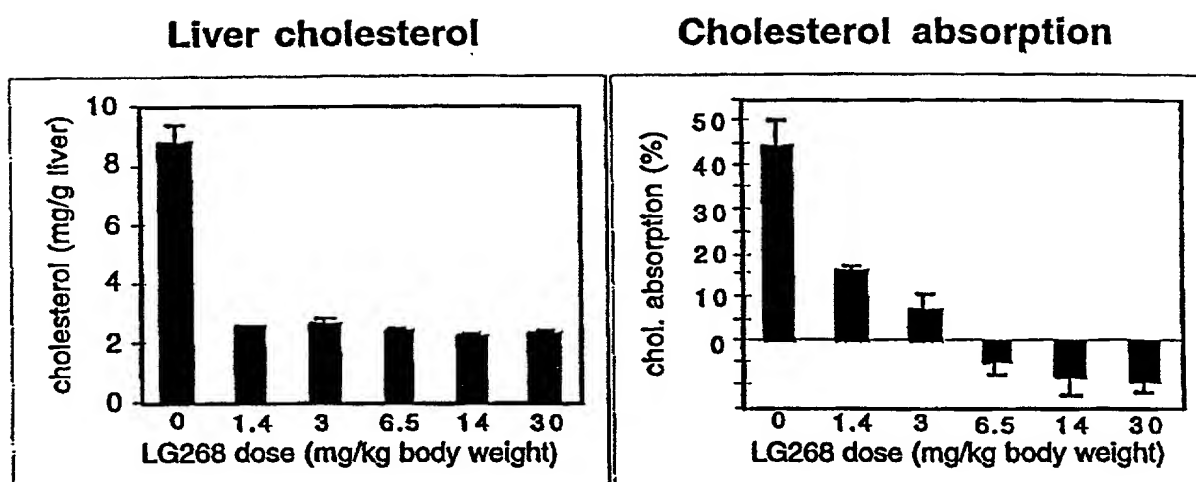


FIG. 4

### Lipid absorption

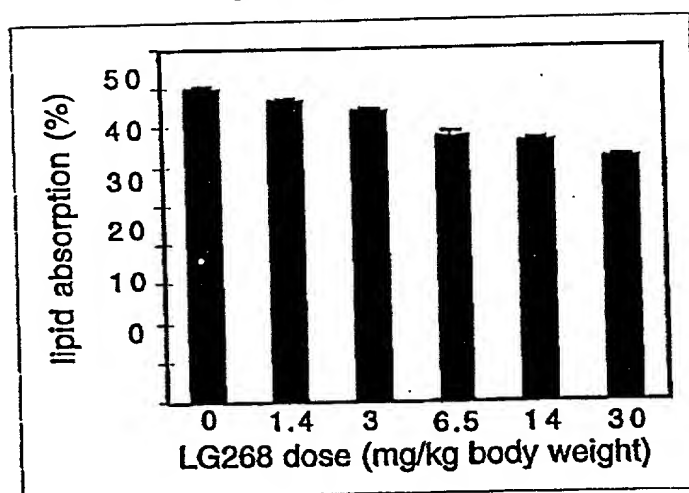


FIG. 5

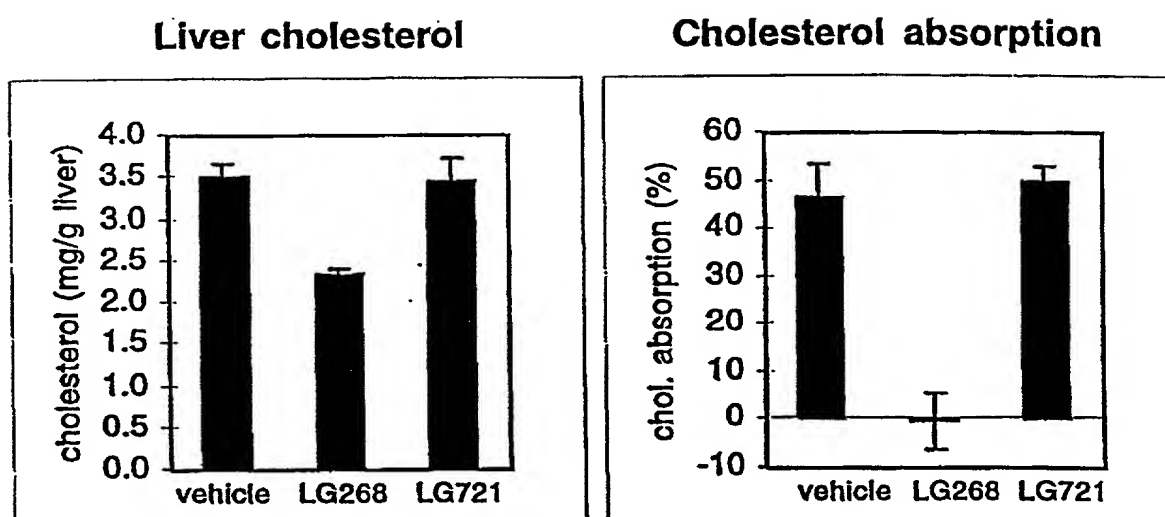


FIG. 6

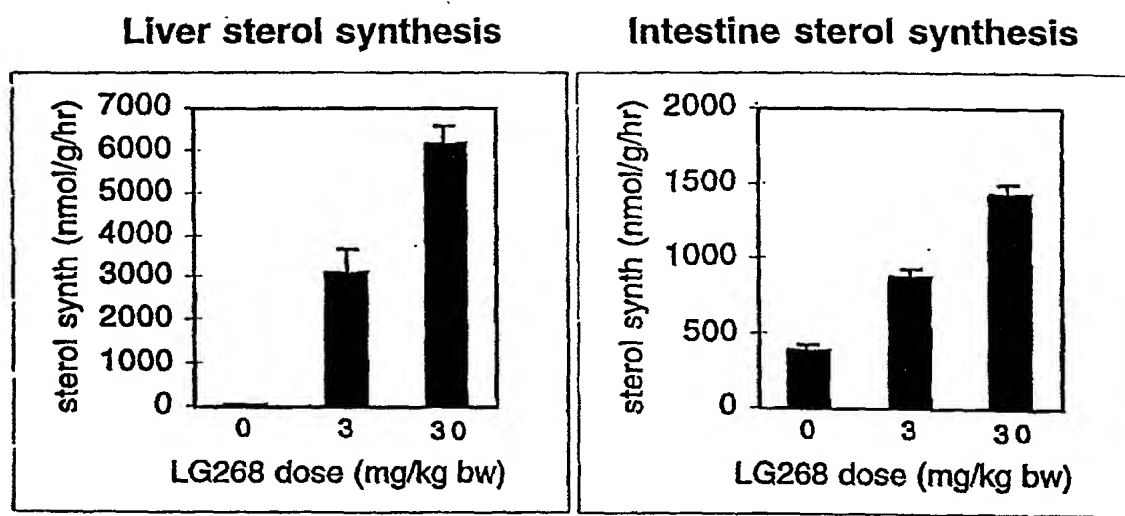


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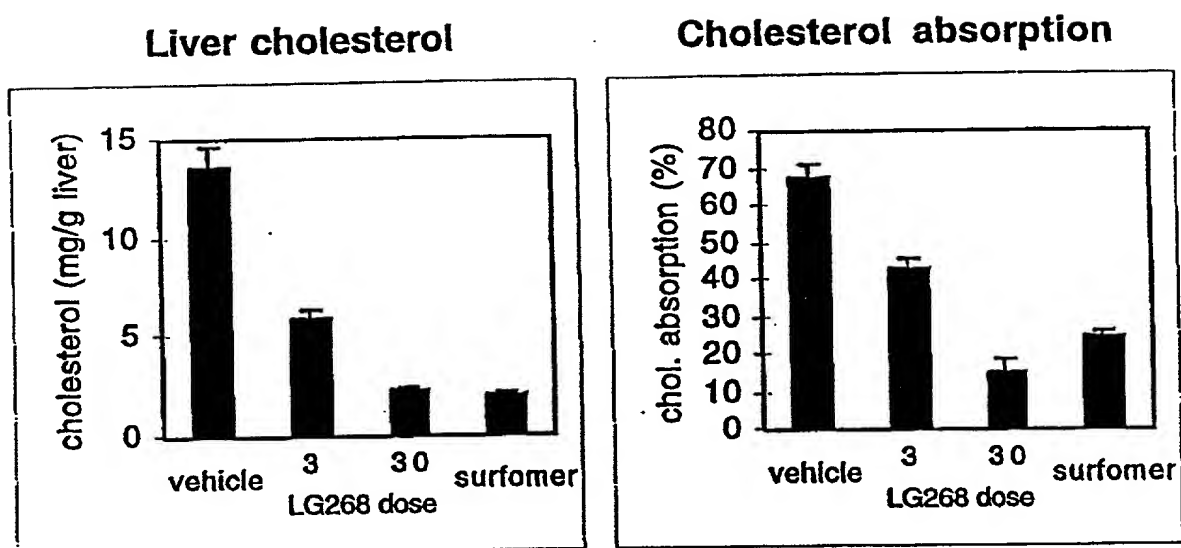


FIG. 8



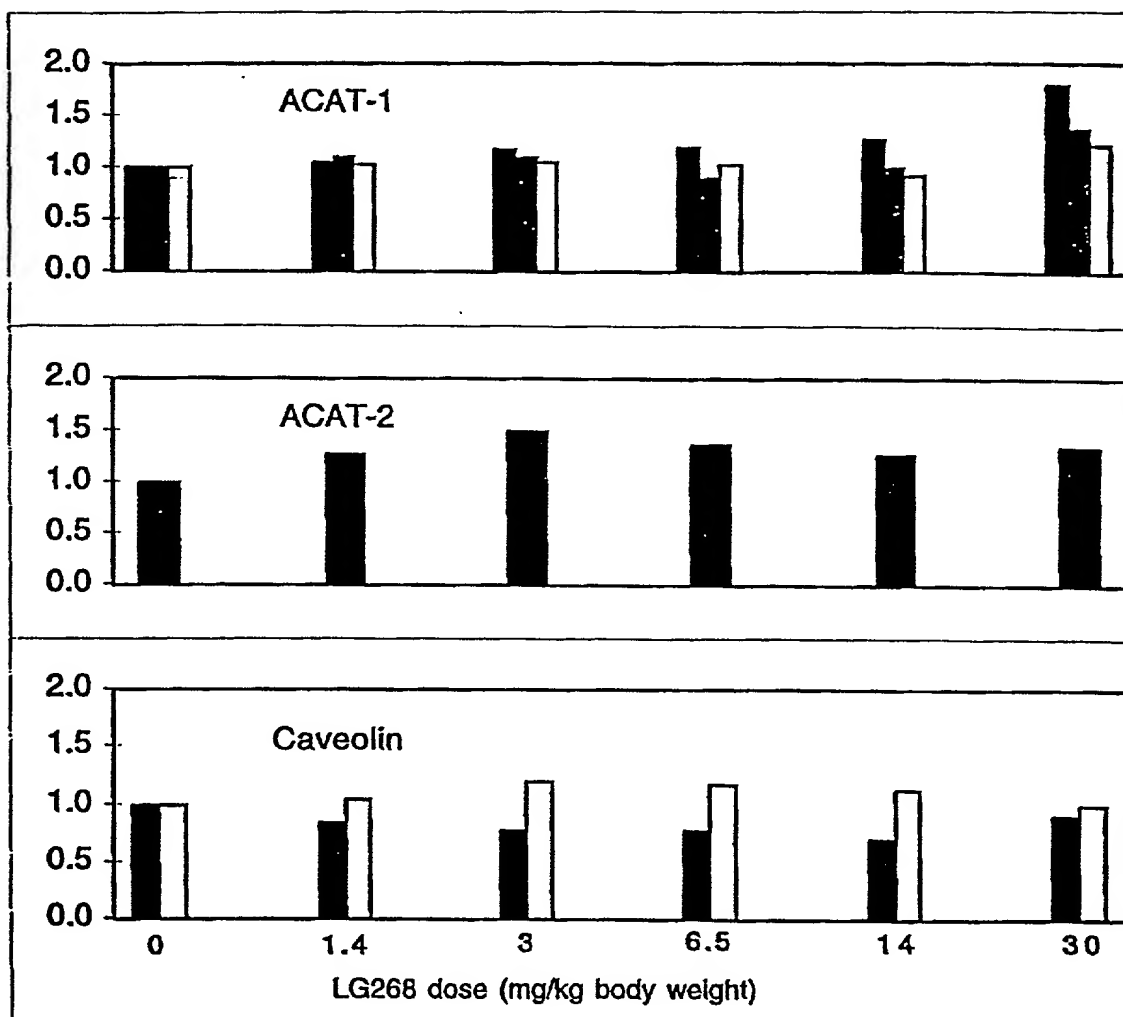


FIG. 9

FIG. 10A

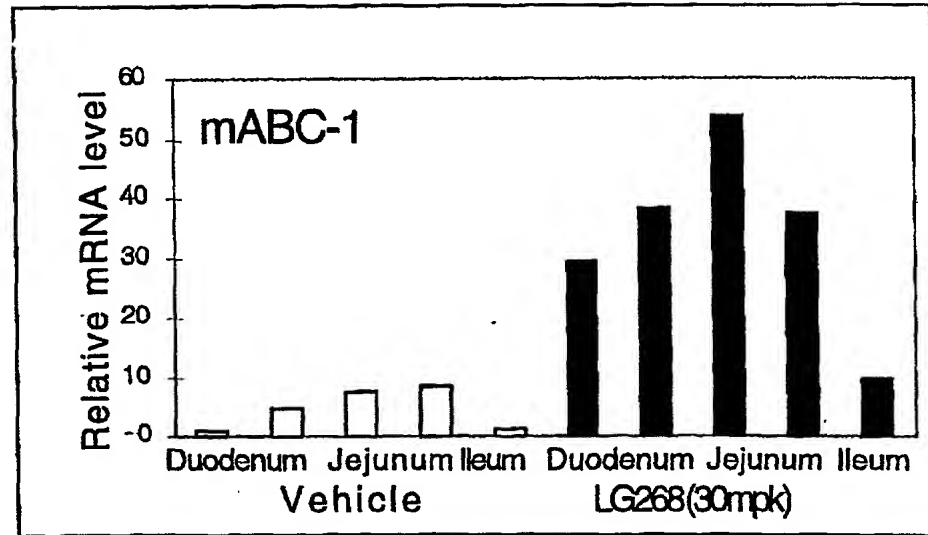
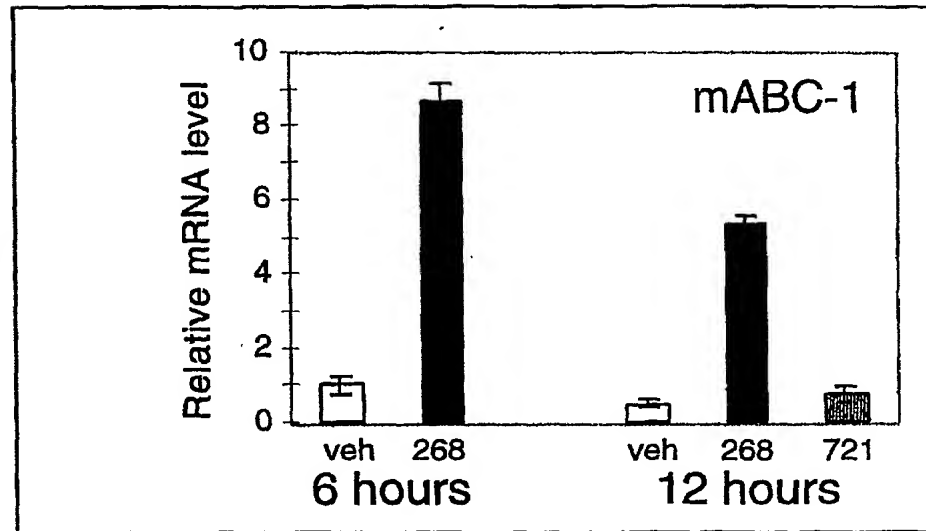


FIG. 10B



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**PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

David J. Mangelsdorf

Joyce J. Repa

Stephen D. Turley

John M. Dietschy

Group Art Unit: Unknown

Examiner: Unknown

Atty. Dkt. No.: UTSD:596/HYL

Serial No.: Unknown

Filed: Unknown

For: COMPOSITIONS AND METHODS OF  
MODULATING CHOLESTEROL  
METABOLISM

STATEMENT AS REQUIRED UNDER 37 C.F.R. § 1.821(f)

**BOX SEQUENCE**

Assistant Commissioner for Patents

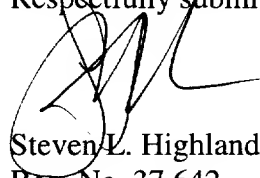
Washington, D.C. 20231

Sir:

Submitted herewith is a computer readable form and a paper copy of the sequence listing of those sequences in the captioned patent application. The computer readable form of the sequence listing is the same as the paper copy of the sequence listing. The sequence information

provided in the Specification is also the same as the sequence listing of the enclosed computer readable and paper forms of the sequence listing.

Respectfully submitted,



Steven L. Highlander  
Reg. No. 37,642  
Attorney for Applicants

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(713) 787-1400

Date: December 10, 1999

SCANNED  
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 REPA, JOYCE J.  
 TURLEY, STEPHEN D.  
 DIETSCHY, JOHN M.

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